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13. SUPPLEMENTARY NOTES

14. ABSTRACT Surveys of individuals with spinal cord injury (SCI) reveal that bladder and sexual dysfunction are among the highest priorities after injury, yet few studies are being done. Our study uses a clinically relevant chronic SCI animal model that is geared toward improving bladder and sexual function post-SCI and thus will promote overall health, independence and self-image. Current attempts at treatments with various drugs and surgeries have unwanted side effects and limited success. Our goal is to provide therapeutic alternatives using body weight supported treadmill training in combination with neurostimulation. We have clinical data from several human SCI study participants that step training can have beneficial outcomes on bladder maintenance, including polyuria (over-production of urine resulting in the need to awaken multiple times during the night to catheterize), as well as erectile function. We envision a therapeutic intervention involving a complement of neurostimulation, drug treatment and some form of at home stepping exercise, as most SCI patients do not have easy access to trained therapists on a daily basis. Thus, the current study examines the effects of locomotor training on bladder and sexual function in a clinically relevant animal model to identify the mechanisms involved. Each part of this study is specifically designed to be applicable to the SCI patient with the intent of taking the results back to the clinical setting (SCI individuals for our human studies are recruited from the Frazier Rehabilitation Institute patient base in collaboration with clinical faculty in the Department of Urology at the University of Louisville School of Medicine).

15. SUBJECT TERMS

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Table of Contents

1) <u>Introduction</u>

Individuals with spinal cord injury (SCI) rank bladder and sexual functions as those that should be given the highest priority in order to enhance quality of life. To develop the most effective therapeutic strategies for improving urogenital function after SCI, an understanding of the mechanisms underlying these functional gains with LT only and with the addition of acute neurostimulation is needed. The current proposal focuses on our continued efforts to optimize the effects of activity dependent plasticity induced by LT after chronic SCI on non-locomotor systems (urogenital function). The aims are as follows: <u>Aim 1</u>: An absence in the night-time rise in anti-diuretic hormone (ADH) levels in SCI subjects and resulting increased urine production leads to autonomic dysreflexia due to bladder over-distention and interrupted sleep, affecting both quality of life and health. In this aim we will determine whether the combination of exercise and pharmacological treatment with desmopressin (an ADH receptor agonist) can reduce polyuria in SCI rats (as measured using metabolic cages). Plasma ADH levels will be determined bi-weekly and for terminal tissue analyses, kidney vasopressin V2 receptor numbers will be quantified and ADH positive cells within the hypothalamus (supraoptic nucleus and paraventricular nucleus) will be counted. <u>Aim 2</u>: Multiple human and animal studies demonstrate that bladder function post-SCI can be improved with electrical stimulation of the pudendal nerve. Since both LT and pudendal stimulation may exert their effects by inducing plasticity of lumbosacral circuitry, the question arises as to whether each affects bladder function through the same or different mechanisms, and whether the influences may be additive. Also, our initial human bladder experiments included testing with LT combined with epidural stimulation. Improvements that were found could be due to indirect activation (close proximity of the electrode array which extends to S1). Thus, Aim 2 will test the effectiveness of both pudendal as well as epidural stimulation on bladder function in both non-trained as well as LT SCI rats. In addition, a non-trained group and a forelimb-only training group (for the alternate possibility of general exercise benefits) will be used as controls. <u>Aim 3 (same rats as in Aim 2)</u>: Given our new data on the benefits of LT on human sexual function, this aim is designed with the goal of (a) validating for future mechanistic studies if LT improves specific sexual reflexes (behavioral assessments throughout training and terminal EMG recordings of the bulbospongiosus muscle) and (b) the effectiveness of neuromodulation. Results from this proposed parallel study in animals will provide further insights to underlying mechanisms from our observations clinically that can come back to be tested in the human model. Benefits from this study will extend to the military as urologic conditions are a burden for Veterans and rates of sexual dysfunction are high in this population.

2) <u>Keywords</u> Bladder, spinal cord injury, cystometry, urodynamics, locomotor training, exercise, vasopressin, ADH, detrusor-sphincter dyssynergia

3) <u>Accomplishments</u>

Major Goals:

Specific Aim 1 (Months 3-22): To determine whether the combination of exercise (forelimb training) and pharmacological treatment with desmopressin can reduce polyuria in SCI rats. SCI groups will include: forelimb-trained (N=12) with placebo pellet, forelimb-trained (N=12) with desmopressin pellet, non-trained harnessed (N=12) with placebo pellet, non-trained harnessed

(N=12) with desmopressin pellet, non-trained left in home cage (N=12) with placebo pellet, and non-trained left in home cage (N=12) with desmopressin pellet.

Specific Aim 2 and 3 (Months 23-36): To test the effectiveness of both pudendal as well as epidural stimulation on bladder function (Aim 2) and to determine if LT improves specific sexual reflexes (behavioral assessments throughout training and terminal EMG recordings of the bulbospongiosus (BS) muscle) and to assess the effectiveness of neuromodulation (Aim 3). SCI groups will include: quadrupedally step trained (LT group; N=12), forelimb only trained (similar to SCI patients rowing or arm crank exercises; N=12), and non-trained in home cage (N=12).

Table 1: SOW (proposed months for 1st year with actual months in parentheses)

Major Task 1: IACUC and ACURO approvals	1 – 2 (1 - 3)
Major Task 2: Experiments for first 18 rats - 3 forelimb-trained with placebo pellet, 3 forelimb-trained with desmopressin pellet, 3 non-trained harnessed with placebo pellet, 3 non-trained harnessed with desmopressin pellet, 3 non-trained left in home cage with placebo pellet, and 3 non-trained left in home cage with desmopressin pellet.	3 – 6 (4 -8)
Milestone(s) Achieved: completion of first 6 groups of 3 rats per group (total 18)	(9-10; Jun ’16)
Major Task 3: Experiments for 2nd group of 18 rats - 3 forelimb-trained with placebo pellet, 3 forelimb-trained with desmopressin pellet, 3 non-trained harnessed with placebo pellet, 3 non-trained harnessed with desmopressin pellet, 3 non-trained left in home cage with placebo pellet, and 3 non-trained left in home cage with desmopressin pellet.	7–10 (11–14)
Milestone(s) Achieved: completion of half Aim 1 rats (36 of 72)	(14-15; Dec‘16)
Major Task 4: Experiments for 3rd group of 18 rats - 3 forelimb-trained with placebo pellet, 3 forelimb-trained with desmopressin pellet, 3 non-trained harnessed with placebo pellet, 3 non-trained harnessed with desmopressin pellet, 3 non-trained left in home cage with placebo pellet, and 3 non-trained left in home cage with desmopressin pellet.	11–14 (16 – 20; Jan‘17)

Research Accomplishments:

Per the statement of work, IACUC and ACURO approvals (**Major Task 1**) were obtained during the initial phase. ACURO approval was given on December 8 and the notice received on December 18. Animals (n=18) for **Major Task 2** were then ordered on the first available date (January 7, 2016 – due to the holiday break). After acclimation and baseline assessments, spinal contusion injuries were done (Jan. 25th & 26th, 2016). The injury parameters for the 18 rats are provided in Table 2 (force and displacement). Four day urine volumes (indicative of degree of bladder dysfunction; larger residual volume indicates greater severity of dysfunction) and two week over-ground locomotor scores (BBB score – scale of 0 to 21 where 21 is normal) were conducted prior to the start

of training (Feb. 8, 2016). These data are also now provided in Table 2. Mid-training and end of training BBB locomotor scores (for left/right hind limbs) are also provided in Table 2.

At the start of training, the 18 rats were randomized into six groups of three each per Major Task 2. Weekly behavioral measures were then conducted. Blood draws were done pre-injury, pre-training (2 weeks post-SCI) and prior to the terminal assessments. Terminal assessments were spread over a few weeks (adding approximately one month to the original SOW timeline) to allow for time-consuming cystometry assessments (late April through May 2016).

Table 2. Injury data, Pre-training Baseline Assessments and BBB locomotor scores

Animal #	Injury Force (kdyn)	Displacement (um)	Bladder Vol. (ml)	Pre-Train (14 day) BBB	Mid-Training BBB	End Training BBB
1	224	1411	4.13	8--8	8--8	8--8
2	214	1499	6.585	8--8	9--9	8--8
3	214	1340	4.8	10--10	10--10	10--10
4	278	1393	4.424	8--8	8--9	8--8
5	219	1552	2.84	10--8	11--11	11--10
6	258	1552	4.09	10--10	9--8	8--8
7	251	1587	3.845	6--0	4--0	6--1
8	221	1446	2.17	8--8	8--8	8--8
9	224	1270	2.49	10--11	11--11	11--11
10	248	1746	3.95	8--8	10--10	11--10
11	352	1675	3.985	8--8	11--11	11--11
12	218	1569	4.205	11--10	8--8	8--8
13	238	1287	3.865	10--10	11--11	11--11
14	221	1411	3.13	10--10	11--11	11--11
15	220	1483	5.07	8--6	9--9	9--9
16	219	1375	3.735	10--10	11--11	12--12
17	220	1111	0.87	8--8	10--10	11--11
18	217	1446	4.13	8--8	12--12	12--12

Blood has been collected via tail vein draw for all subjects for several time points during the experiment (pre, post injury and terminal) and following centrifugation it is stored at -20 degrees for subsequent purification and further analysis. Blood levels of ADH (vasopressin) and corticosterone have been measured to date along with urinary norepinephrine for both the pre-injury and pre-training/post-injury samples. These data are presented in Table 3. Urine collected from 24 hour metabolic cage sessions has also been centrifuged and aliquoted and is stored at -20 degrees for subsequent analysis. This urine collection includes collection from animals D1-18 during the cycle of desmopressin or placebo injections. We are currently awaiting the arrival of desmopressin ELISA kits so that we can begin to test these samples to quantify levels of desmopressin in subjects who received the injections.

The metabolic cage data for pre- and post-desmopressin or placebo measurements is provided in Table 4a and 4b. The cystometry data from the terminal study are presented in Table 5 (ICI – inter-contraction interval; MAC – maximal amplitude of contraction; voiding efficiency) along with the final terminal bladder, kidney and prostate weights.

<u>Table 3</u>. Blood Sampling Assessments

	Plasma AVP levels (pg/ml)		CORT/Cre ratio		Norepinephrine/Cre ratio	
Animal #	Pre-injury	Post-injury	Pre-injury	Post-injury	Pre-injury	Post-injury
1	2.18826	6.48592	102.96511	125.54389	42.00252	11.44464
2	5.60028	5.06574	112.27129	5.536624	6.49632	0.815208
3	5.6676	8.80268	24.66562	48.537963	0	6.769104
4	6.57698	9.29568	28.549814	22.48745	7.10508	2.31144
5	7.19048	7.48694	43.818272	37.81932	9.687168	7.447248
6	10.4899	6.9708	29.874154	27.42995	9.5298	6.052944
7	7.19302	5.77924	8.808748	115.21844	2.172654	0
8	5.80106	6.36306	52.016904	42.195712	10.20274	4.192872
9	4.74362	7.3866	28.599394	41.483942	9.216912	6.727848
10	10.9323	6.57698	29.970386	101.27101	6.118512	33.76248
11	6.6115	7.8031	49.616654	149.23988	7.528296	41.0328
12	8.61898	8.21712	65.344088	27.094316	9.616224	1.397304
13	6.77062	6.67416	152.19766	26.302604	31.56588	6.366456
14	8.15226	3.68816	26.052578	88.919058	4.97076	9.775848
15	12.77394	5.06234	30.793984	50.51322	6.357528	0
16	7.01016	6.03084	30.52046	129.88312	5.71968	37.8492
17	6.15066	4.7357	22.071826	28.810926	5.590824	7.526232
18	4.69024	4.4725	18.540064	23.249362	4.884672	8.33508

<u>Table 4a</u>. Metabolic Cage Data

Urine volumes (ml)

Rat #	Pre-injection	Day 1 injection	Day 2 injection	Day 3 injection	3 days post injection
1	14.2	13.5	11.3	14.5	17.2
2	15.6	18	19.5	19.3	29.5
3	16	16	15	12.3	15.6
4	23.8	20.1	21	29	27.4
5	14.4	11.4	14.5	17.7	19.1
6	12.2	15	20.4	26.4	23
7	9	13.9	9	12.6	13.5
8	13.2	13.2	11	15	16.5
9	14.5	14.5	6.2	13.8	16.4
10	20.3	8.5	12.7	11.6	20
11	15.3	23.3	19.3	18.3	17
12	15.5	14.1	19.5	19.1	22.6
13	18.3	12	11.2	12	18.5
14	13.3	12.3	12.3	15	17.2
15	14.6	15.5	17	17	23.2

16	32	23.8	16	12.1	26.6
17	15.1	9	16	18	19
18	12.2	11	12	11.2	18

Table 4b. Metabolic Cage Data

Drink totals (ml)

Rat #	Pre-injection	Day 1 injection	Day 2 injection	Day 3 injection	3 days post injection
1	32.54	26.48	34.6	34.9	38.62
2	48.98	43.72	40.44	51.8	50.5
3	39.48	39.38	29.38	35.78	32.74
4	48.68	40.68	48.72	51.3	55.14
5	45.88	28.84	41.1	41.82	36.12
6	48.46	50.16	56.38	52.38	51.6
7	23.2	25.18	23.28	23.24	24.82
8	39.72	37.06	38.78	34.46	37.38
9	35.56	36.56	6	39.4	27.76
10	47.74	28.24	29.4	31.14	45.26
11	42.08	45.76	46.78	50.68	52.6
12	45.92	44.06	51.14	42.2	41.92
13	44.08	38.5	27.44	22.28	42.04
14	31.62	34.4	38.02	44.08	47.58
15	45.06	35.86	37.32	35.98	38.3
16	61.02	40.1	23.36	29.5	51.9
17	36.74	26.54	46.38	42.74	39.1
18	47.46	44.94	45.24	44.62	46.78

Table 5. Terminal Study: Cystometry Data, Bladder Weights, and other organ weights

Rat #	ICI (secs)	MAC (mmHg)	Void efficiency (%)	Bladder weight (g)	Kidney (g)	Prostate (g)
1	41.26	47.7	84	0.42	2.14	0.575
2	257	53.9	80	0.31	1.745	0.62
3	161	34.7	110	0.195	1.68	0.52
4	239.1	33.41	87	0.31	2.115	0.66
5	175	33.98	71	0.245	2.25	0.865
6	387.3	35.02	102	0.32	1.87	0.99
7	334.5	43.12	89	0.32	2.25	0.385
8	177.45	48.35	93	0.33	1.525	0.705
9	70.4	43.07	86	0.275	1.645	0.27
10	117.87	34.79	71	0.255	2.22	0.79
11	277.4	38.74	99	0.325	1.875	0.78

12	181.8	32.62	96	0.28	1.995	1.08
13	98.1	56.28	88	0.23	2.03	0.66
14	61.6	33.02	87	0.285	1.31	0.56
15	606.1	42.96	79	0.39	2.35	0.96
16	106.3	28.55	48	0.415	1.945	0.74
17	283.2	45.8	106	0.31	1.65	0.61
18	248.2	37.1	131	0.28	1.58	0.945

Lesion reconstruction is currently in progress. Thus far, the tissues have all been cut, mounted and stained for analysis. Initial measurement of lesion epicenter area is presented in Table 6 (% white matter sparing [WMS]; % grey matter sparing [GMS]). Lesion volume assessments are still in progress.

Table 6. Lesion Reconstruction Data: Epicenter Area

Animal	WMS%	GMS%
1	17.4	0
2	20.9	1.41
3	26	1.79
4	17.1	0
5	18.5	0
6	17.9	1.63
7	7.42	0
8	11.45	1.22
9	10.3	4.9
10	25.4	5.24
11	9.75	0
12	15.62	1.65
13	13.27	0
14	5.97	1
15	15.4	0
16	11.77	0
17	17.7	0
18	15.12	0

The first 18 rats represent 3 animals in each of six groups (per SOW). These data are not sorted by training group or decoded as this will only be done after all the Aim 1 group data has been collected (to maintain blindness of investigators conducting the tissue and data analysis). The second sets of 18 rats are currently in their post-injury training rehabilitation phase. The injury parameters for those 18 rats (Animal #'s 19-36) are provided in Table 7 (force and displacement). Four day urine volumes (indicative of degree of bladder dysfunction; larger residual volume indicates greater severity of dysfunction) and two week over-ground locomotor scores (BBB score – scale of 0 to 21 where 21 is normal) were once again conducted prior to the start of training. These data are also now provided in Table 7. At the start of training, the 18 rats were randomized into six groups of three each per Major Task 3. Weekly behavioral measures are currently being conducted.

Table 7. Injury data, Pre-training Baseline Assessments and BBB locomotor scores

Animal Number	Bladder Vol. (ml)	Injury Force (kdyne)	Displacement (um)	BBB (14 day) Score
19	1.54	221	1499	8--8
20	5.675	217	1464	8--8
21	0.74	215	1464	8--8
22	4.42	214	1411	10--10
23	3.87	245	1746	8--10
24	0.995	220	1322	11--11
25	3.61	269	1499	8--7
26	3.885	244	1481	8--8
27	1.72	220	1411	11--11
28	1.915	220	1587	9--9
29	2	227	1658	10--10
30	1.49	224	1516	8--8
31	1.37	227	1658	7--7
32	2.33	224	1411	9--9
33	3.63	216	1375	8--8
34	2.515	324	1534	10--10
35	1.595	267	1411	8--8
36	1.04	218	1393	8--8

Opportunities for training and professional development:

Training is being provided to a sophomore undergraduate Biology Major who is assisting with the training of animals. Likewise, funding of a postdoctoral associate and graduate student who are participating in the study have opportunities to attend local seminars and journal club in the Kentucky Spinal Cord Injury Research Center as part of their professional development.

Disseminated to communities of interest:
Nothing to report

Plans during the next reporting period to accomplish the goals:

In the first part of Year 2 (current), Major Task 3 will be completed; i.e., training and terminal assessments for the second groups of animals, numbers 19 through 36. The terminal assessments will be spread through mid-November into December as each animal testing is time consuming. On the first Thursday in January, the next group of 18 rats will be ordered (3 more per group). Spinal cord injuries will be made and the rats trained and tested. Terminal assessments will be done mid-April into May. At this time, we will assess whether we have sufficient data for Aim 1 to move onto Aims 2 and 3 or if one more set of 18 rats are needed (depending on animal loss and if inclusion/exclusion criteria are met such as lesion severity). Twelve rats per group (6 groups) was proposed with the goal of 10 per group.

Major Task 4: Experiments for 3rd group of 18 rats - 3 forelimb-trained with placebo pellet, 3 forelimb-trained with desmopressin pellet, 3 non-trained harnessed with placebo pellet, 3 non-trained harnessed with desmopressin pellet, 3 non-trained left in home cage with placebo pellet, and 3 non-trained left in home cage with desmopressin pellet.	
Subtask 1: Pre-injury baseline assessments (once a week for two weeks): • Locomotor assessment using 21-point BBB scale • Voiding volume, voiding frequency, water and food intake (metabolic cages - CLAMS) • Home cage activity (Opto M3 – incorporated into CLAMS system) • Blood sampling (one sample – for assessing ADH, corticosterone and norepinephrine levels)	16
Subtask 2: Contusion injuries then post-injury baseline assessment (once a week for two weeks): • Locomotor assessment using 21-point BBB scale • Voiding volume, voiding frequency, water and food intake (metabolic cages - CLAMS) • Home cage activity (Opto M3 – incorporated into CLAMS system) • Blood sampling (one sample)	16
Subtask 3: 4 weeks of daily training on a treadmill beginning two weeks post-injury (weekly assessments except *) • Locomotor assessment using 21-point BBB scale • Voiding volume, voiding frequency, water and food intake (metabolic cages - CLAMS) • Home cage activity (Opto M3 – incorporated into CLAMS system) • Blood sampling (* every other week)	17
Subtask 4: Insertion of desmopressin and placebo pellets after end of 4 weeks of training. • Locomotor assessment using 21-point BBB scale • Voiding volume, voiding frequency, water and food intake (metabolic cages - CLAMS) • Home cage activity (Opto M3 – incorporated into CLAMS system) • Blood sampling (every other week)	18
Subtask 5: 4 more weeks of daily training on a treadmill. • Locomotor assessment using 21-point BBB scale • Voiding volume, voiding frequency, water and food intake (metabolic cages - CLAMS) • Home cage activity (Opto M3 – incorporated into CLAMS system) • Blood sampling (every other week)	18
Subtask 6: Terminal assessments • Cystometry to evaluate voiding efficiency, bladder hyper-reflexia, and detrusor-sphincter dyssynergia • Blood sampling • Tissue removal (kidneys, brain region containing the hypothalamus, spinal cord lesion epicenter)	19-20

4) <u>Impact</u>

Impact on the development of the principal discipline(s) of the project:
Nothing to report

Impact on other disciplines:
Nothing to report

Impact on technology transfer:
Nothing to report

Impact on society beyond science and technology:
Nothing to report

5) <u>Changes and/or Problems</u>

A slight modification from our initial study design was approved by the University of Louisville IACUC on 8/18/16 and the amendment subsequently approved by ACURO on 8/23/16. The change involved delivery method of the desmopressin and placebo treatment. Originally, it was to be delivered subcutaneously as an implanted slow release pellet. This procedure needed to be modified in light of evidence we obtained that the pellets we purchased were not delivering the intended drug dosages that were needed. We are now delivering the desmopressin or placebo via daily subcutaneous injection (standard delivery method).

6) <u>Products</u>

Two articles were published this past year on a related topic from our previous DOD funded study for which this is a continuation. Although specific data from the current study was not presented, the concepts and previous animal and human data regarding the impact of training on urogenital function was presented in several formats as summarized below.

<u>Publications, Abstract, Presentations</u>

Peer-reviewed Scientific Journal Articles to date:

Hubscher, C.H., Montgomery, L.R., Fell, J.D., Armstrong, J.E., Poudyal, P., Herrity, A.N. and Harkema, S.J. (2016) Effects of exercise training on urinary tract function after spinal cord injury. AJP – Renal. 310(11):F1258-68. (PMID:26984956).

Ward, P.J., Herrity, A.N., Harkema, S.J. and Hubscher, C.H. (2016) Training-induced functional gains following spinal cord injury. Neural Plasticity. http://dx.doi.org/10.1155/2016/1307694.

Abstracts to date:

Hubscher, C.H., Herrity A.N., Montgomery, L.R., Willhite, A.M., Angeli, C.A., and Harkema S.J. (2015) Task-specific training-based rehabilitation improves bladder outcomes following human spinal cord injury. *2015 Abstract Viewer/Itinerary Planner.* Chicago IL: Society for Neuroscience.

Montgomery, L.R., Herrity A.N., Harkema S.J., and Hubscher, C.H. Exercise-dependent modulation of neuro-urological health following spinal cord injury. *2015 Abstract Viewer/Itinerary Planner.* Chicago IL: Society for Neuroscience.

Hubscher, C.H., Montgomery L., Fell J., Poudyal P., Armstrong J.A., Herrity A.N., Harkema S.J. (2015) Effects of physical activity on urinary tract function after spinal cord injury. Open discussion poster presentation in Neurourology: Basic Science Category. International Continence Society Meeting, Montreal, Canada.

Presentations to date:

The 34th Annual Neurotrauma Symposium. Speaker in session entitled "Management of autonomic dysfunction after spinal cord injury". Lexington Convention Center, Lexington Kentucky; June 2016.

"Translational studies examining the impact of locomotor training on urogenital and bowel function after spinal cord injury." Program in Neuroscience, Department of Psychology, Florida State University, Tallahassee, Florida; April 2016.

"Translational studies of locomotor training after spinal cord injury: improvements in urologic function." Department of Neural and Behavioral Sciences, Penn State University School of Medicine, Hershey, Pennsylvania; March 2016.

<u>Inventions, Patents and Licenses</u>

None to report

7) <u>Participants & other collaborating organizations</u>

Individuals who have worked on the project:

Name:	Charles Hubscher, Ph.D.
Project Role:	P.I.
Effort:	20%
Contribution to Project:	Oversees all aspects of the project.
Name:	James Armstrong
Project Role:	Senior Research Technician
Effort:	40%
Contribution to Project:	Involved with all aspects of the project, including ordering of supplies and animals.
Name:	Jason Fell
Project Role:	Research Technician
Effort:	40%
Contribution to Project:	Involved with animal training and terminal studies, perfusions, spinal lesion removal, reconstructions, and histological analyses.
Name:	Lynnette Montgomery, Ph.D.
Project Role:	Postdoctoral Fellow
Effort:	30%
Contribution to Project:	Involved with all aspects related to the desmopressin treatment, including blood draws and analyses, as well as kidney and hypothalamic assessments.

Cont'd:

Name:	Casey Steadman, M.S.
Project Role:	Graduate Student
Effort:	50%
Contribution to Project:	Involved with training, metabolic cage and sexual reflex assessments.
Name:	David Heng
Project Role:	Temporary worker (undergraduate student)
Effort:	100% (of a part-time position)
Contribution to Project:	Involved with training of the animals.

Changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period:
Nothing to report

Other organizations involved as partners:
Nothing to report

8) <u>Special Reporting Requirements</u>

Nothing to report

9) <u>Appendices</u>

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Ward, P.J., Herrity, A.N., Harkema, S.J. and Hubscher, C.H. (2016) Training-induced functional gains following spinal cord injury. Neural Plasticity. http://dx.doi.org/10.1155/2016/1307694.

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Effects of exercise training on urinary tract function after spinal cord injury

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Hubscher CH, Montgomery LR, Fell JD, Armstrong JE, Poudyal P, Herrity AN, Harkema SJ. Effects of exercise training on urinary tract function after spinal cord injury. *Am J Physiol Renal Physiol* 310: F1258–F1268, 2016. First published March 16, 2016; doi:10.1152/ajprenal.00557.2015.—Spinal cord injury (SCI) causes dramatic changes in the quality of life, including coping with bladder dysfunction which requires repeated daily and nightly catheterizations. Our laboratory has recently demonstrated in a rat SCI model that repetitive sensory information generated through task-specific stepping and/or loading can improve nonlocomotor functions, including bladder function (Ward PJ, Herrity AN, Smith RR, Willhite A, Harrison BJ, Petruska JC, Harkema SJ, Hubscher CH. *J Neurotrauma* 31: 819–833, 2014). To target potential underlying mechanisms, the current study included a forelimb-only exercise group to ascertain whether improvements may be attributed to general activity effects that impact target organ-neural interactions or to plasticity of the lumbosacral circuitry that receives convergent somatovisceral inputs. Male Wistar rats received a T9 contusion injury and were randomly assigned to three groups 2 wk postinjury: quadrupedal locomotion, forelimb exercise, or a nontrained group. Throughout the study (including preinjury), all animals were placed in metabolic cages once a week for 24 h to monitor water intake and urine output. Following the 10-wk period of daily 1-h treadmill training, awake cystometry data were collected and bladder and kidney tissue harvested for analysis. Metabolic cage frequency-volume measurements of voiding and cystometry reveal an impact of exercise training on multiple SCI-induced impairments related to various aspects of urinary tract function. Improvements in both the quadrupedal and forelimb-trained groups implicate underlying mechanisms beyond repetitive sensory information from the hindlimbs driving spinal network excitability of the lumbosacral urogenital neural circuitry. Furthermore, the impact of exercise training on the upper urinary tract (kidney) underscores the health benefit of activity-based training on the entire urinary system within the SCI population.

bladder; kidney; locomotor training; contusion

IMPROVING BLADDER DEFICITS is among the areas of highest priority following spinal cord injury (SCI), as urinary tract impairment has an enormous impact on the quality of life (2, 3, 26). Life-long urological care is required for SCI individuals, yet most efforts treat symptoms but do not improve intrinsic function (68, 80). Bladder management requires intermittent catheterization throughout the day/night to avoid incontinence, bladder overdistention (which can create high pressure and reflux to the kidneys), inflammation, infections, and autonomic dysreflexia.

Despite bladder dysfunction being a high priority for SCI individuals, the focus of health care professionals is on rehabilitation aimed at optimizing mobility and the remaining musculoskeletal function. Locomotor training (LT) has emerged as a safe and effective therapy for post-SCI motor deficits with many benefits (cardiovascular function, strength, mobility) (9, 23, 43, 52, 84). Recent animal studies, however, have shown that LT post-SCI also improves bladder function (46, 90), a finding consistent with a few reports from human SCI studies (42, 48, 75). For example, a recent study from our laboratory (90) has shown, using a spinal contusion model in adult male rats, functional gains of lower urinary tract function as assessed with terminal urodynamic measures after 12 wk of daily LT for a period of 60 min/day. The interaction of lower limb musculature with the bladder and its sphincter has been observed sporadically over the years, as far back as 1933, in both humans and animal studies (20, 53, 74). Flexor and extensor reflexes can be modulated by the state of bladder filling and voiding in normal humans and those with central nervous system damage (64). In humans with spasticity, the general pattern is that detrusor contractions precede limb flexor spasms (69). This vesicosomatic relationship involving lumbosacral reflex circuitries could contribute to the enhancement of bladder function with LT.

The multisystem functional gains with task-specific training have generated multiple novel hypotheses regarding potential underlying mechanisms. With respect to the improvements in bladder function with 60 min/day of stepping on a treadmill using body weight support and manual facilitation in a natural position (90), the current study was designed to expand upon our initial findings to include *1*) non-weight-bearing stepping with a forelimbs-only exercise group; *2*) collection of weekly metabolic cage data to monitor SCI-induced persistent polyuria (overproduction of urine); and *3*) further tissue assessments that include the structural integrity of the bladder wall and the impact of training on the upper urinary tract (kidneys).

The bladder wall itself has been shown to be more compliant after spinal transection, and the extracellular matrix components largely determine its mechanical properties (36). Collagen and elastin, two major connective tissue proteins, provide tensile strength and elasticity and are implicated as being directly responsible for the mechanical changes in the bladder wall of SCI rats (83), so their quantification will give a more accurate picture of potential bladder composition remodeling.

Long-standing detrusor sphincter dyssynergia with chronic SCI, even with careful bladder management, may lead to vesicoureteral reflux due to high bladder pressures from overdistention and complications that include kidney infections, pyelonephritis, and hydronephrosis (4, 13, 59). Bladder infections from multiple daily catheterizations may also spread to the kidney. Chronic kidney disease is highly prevalent in the SCI population, with complications that include decreased

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glomerular filtration rate and renal plasma flow (30, 60, 73). For the current study, the expression of two proteins indicative of tissue turnover in the kidneys, transforming growth factor-β (TGF-β; a fibrogenic growth factor implicated in the pathogenesis of renal scarring) and cluster of differentiation molecule 11b (CD11b; an adhesion molecule which promotes cell-cell adhesion between leucocytes and leucocyte-endothelial cells in inflammation), were assessed to determine whether improved bladder function with LT could also reduce the kidney's susceptibility to post-SCI complications. TGF-β promotes fibrogenesis, cell apoptosis, and tissue healing and suppresses excess cellular proliferation, differentiation, and immunity (66). Although increased expression of TGF-β is considered a valuable marker in determining fibrosis with kidney disease (1, 11, 38, 94, 95), exacerbation of the immune response or autoimmunity has been reported in association with the absence or decreased expression of TGF-β (37), and the immune system is known to be impacted in persons with SCI (61). Also, damage to kidney tissue in ischemia-reperfusion injury can be mitigated by blocking CD11b (71) as it contributes to epithelial injury, inflammation, and fibrosis (32).

METHODS

All experimental procedures were conducted according to National Institutes of Health guidelines, and protocols were approved by the Institutional Animal Care and Use Committee at the University of Louisville School of Medicine.

A total of 55 adult male Wistar rats (Harlan Sprague Dawley, Indianapolis, IN), weighing initially ~250 g, were individually housed in an animal room with a 12:12-h light-dark cycle. They had ad libitum access to water and food (Laboratory Rodent Diet).

SCI

The Infinite Horizon (IH) impactor device (Precision Systems and Instrumentation, Fairfax Station, VA) was used to make a clinically relevant contusion injury (225 kdyne) at the T9 spinal level of 48 rats (7 additional rats served as sham surgical controls). This impact produces a moderate to severe incomplete SCI. Procedures for SCI followed our previously published protocols (41, 49, 50, 91). Briefly, animals were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine [10 mg/kg intraperitoneally (ip)] and 0.1-ml supplemental doses were given ip as needed to maintain a deep surgical level of anesthesia. Vital sign monitoring included heart rate with a rodent stethoscope, respiratory rate, and ventilator status with a small animal oximeter (Starr Life Sciences), body temperature with a rectal thermistor, and anesthetic depth with corneal, palpebral, pedal, and pinna reflexes as well as tail pinch.

For the spinal contusion, the cord was exposed at the T9 level via removal of the overlying vertebral lamina, and the IH impactor force-driven device (76) was used to make the contusions (no dwell time). After the injury, a piece of thrombin-soaked Gelfoam was placed into the vertebral defect, the surrounding musculature and subcutaneous tissue were sutured in layers with 4-0 monofilament, and the skin was closed with Michel clips (removed 7 days po). All rats were injected subcutaneously with 0.5 ml of dual penicillin (penicillin G coupled with Procaine, PenJect, Butler Schein, Columbus, OH) as a general prophylactic and recovered in a temperature-controlled environment. In the days immediately following SCI, all animals were also given subcutaneous injections of ketoprofen (Ketofen, 2.5 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) for analgesia (twice a day for 2 days) and 5 mg/kg gentamicin (Genta-Fuse, Butler Schein, Dublin, OH) to prevent bladder infections (once per day for 5 days).

The urinary bladder was initially emptied by manual crede every 8 h until the micturition reflex occurred automatically (spontaneously triggered by contact with cage bedding or otherwise without caretaker assistance), 6–10 days after injury (29, 51). Residual urine was collected and measured three times over a 24-h period for every rat on the days immediately postinjury and recorded in the animals' surgery recovery log. Only the maximum single daily urine volume (typically the AM volume after lights on, i.e., after the active phase during which more water tends to be consumed) was used for analysis as this value is most reflective of the largest bladder capacity. Note that the amounts obtained at a 3- to 4-day time point are reflective of the initial peak bladder dysfunction immediately post-SCI with higher volumes indicative of a more severe injury (91). These data along with a 2-wk time point score of overground locomotion using the 21-point Basso-Beattie-Bresnahan (BBB) open-field locomotor test (6) (as previously described in detail) (90, 91) were set aside for end-of-study post hoc analysis to check for pretraining group differences in terms of lesion severity. Training was initiated 14 days postinjury after animals were randomly divided into three study groups (as described below). This delayed time frame was chosen because *1*) rehabilitation efforts initiated too early after SCI have been shown to be detrimental as they can exacerbate secondary injury cascades (79) and *2*) 2 wk are beyond the period of SCI-induced plasticity of sacral parasympathetic bladder reflex pathways (18, 19, 86) which can occur up to 10 days (29, 51).

Treadmill Training

SCI rats were randomly divided into 3 groups of 16 each 2 wk postinjury: two groups followed a step-training regimen over a treadmill belt assisted by a body weight support system (Exer-3R treadmill, Columbus Instruments) (90, 91), and a nontrained control group used no body support. All groups were harnessed with a cloth rodent vest (Robomedica, Mission Viejo, CA) with spring scales clipped onto both ends of the vest for weight support as needed. The quadrupedal-trained group (n = 16; SCI+QT) stepped on the treadmill with all four limbs, bearing weight on the hindlimbs with manual facilitation below the level of injury (90), with the spring scales self-adjusted to allow for quadrupedal stepping and complete paw placement. The forelimb-only trained group (n = 16; SCI+FT) had their hindlimbs slightly elevated from the surface of the treadmill using the spring scale (non-weight bearing; similar to arm crank exercise in human SCI) so that they only stepped with their forelimbs. The nontrained control group (n = 16; SCI) was also harnessed on the treadmill for the equivalent 1-h time frame but was not stepped. Seven additional rats, used as noninjured controls (surgical shams: spinal cord exposed like the other groups but no contusion), were handled weekly but remained in their home cages for the duration of the study.

Given the time necessary for the daily step training of each rat, the current study was done in 4 groups of 12 rats each over a period of ~2 yr. Each set of 12 comprised 4 SCI+QT, 4 SCI+FT, and 4 SCI only control rats. In a given hour session, one SCI+QT and one SCI+FT rat were on the 15-inch-wide treadmill belt with one nontrained SCI rat in a harness next to it. Thus 4 h of training were done in total per day, with 15-min breaks every hour between groups. Each set of 12 rats took part in the study for a total of ~14 wk (acclimation period, preinjury testing, SCI, 10 wk of training/testing starting 2 wk post-SCI, awake cystometry at the end of training immediately before euthanasia and tissue removal). A time line is provided in Fig. 1.

Before SCI and post-SCI/pretraining, all rats were exposed to the treadmill environment for at least two half-hour sessions. Training was initiated on schedule at 14 days after injury for all rats, given that there were no signs of infection or other complications arising from the surgery (a rare occurrence). The SCI+QT and SCI+FT groups of animals were at first placed on the treadmill in the harness vest for 20 min, beginning at a slow speed. The session time was gradually increased from 20 min to the target of 58 min over the course of the first 7 training days as described previously (90). All rats on the

EXPERIMENTAL TIMELINE																	
	WEEK 0-Pre-injury				WEEKS 1-2										WEEKS 3-12	WEEK 12	
	ACC	ACC	ACC	MC	SCI	REC	RUC	RUC	RUC	REC		MC	REC	BBB and MC	TT and MC	AC	TR
Days	-7	-6	-5	-4 through -1	0	1	2	3	4	5	6	7	8 through 13	14	14 through 76	77 through 84	
	Baseline Measurements				Post-Injury Pre-Training Measurements										TT & Voiding Activity	Terminal Experiments	
Animals Arrive	Acclimation Period: Handling and Exposure of Animals to Treadmill (ACC)			Metabolic Cage Data Collection (MC)	Spinal Cord Injury (SCI)	Recovery (REC)	Residual Urine Collection (RUC)			REC		MC	REC	BBB Testing and MC	Daily Treadmill Training (TT), Groups: (SCI, SCI+QT, SCI+FL); Weekly MC	Awake Cystometry (AC)	Tissue Removal (TR)

Fig. 1. Timeline. Experimental procedures and treadmill training are indicated relative to the time when the spinal cord injury (SCI) was made (*day 0*). See the text for additional definitions.

treadmill were monitored by an experienced investigator at all times. However, to help provide adequate afferent feedback related to stepping for the SCI+QT group (similar procedure done with locomotor training in humans), the experimenter manually assisted for plantar paw placements with full toe extension and no ankle rotation. Independent stepping was encouraged when the rats had achieved better coordination, stability, and absence of hindlimb dragging. Although sessions are terminated early for any animals showing signs of stress (for example, diarrhea, porphyrin stains in eyes, or irregular breathing pattern) or a session skipped if any abrasions from training were observed on the paw or skin (until healed, as noxious input can interfere with spinal learning) (39), there were no such instances in the current study.

Voiding Behavior

Voiding behavior was assessed weekly with a six-station Columbus Instruments Comprehensive Lab Animal Monitoring System (CLAMS). The CLAMS unit and corresponding software (Oxymax for Windows, version 4.83) was used to collect 24-h urination data and food and water intake for all groups of rats. Animals were placed in cages once a week throughout the experiment (including preinjury, the 2 wk post-SCI, and throughout the 10-wk training period) for 24-h data collection periods with food and water ad libitum. Following each 24-h testing session, each metabolic cage was disassembled, cleaned, and reassembled for the next session.

End of Study Awake Cystometry

Filling cystometry (nonstop transvesical) experiments were conducted in conscious rats (see Refs. 92 and 93 for justification) after ~10 wk of training (or equivalent time frame) for all groups. For catheter placement (47, 54, 63), a 1.5-cm midline abdominal incision was made under brief gas anesthesia (2% isoflurane). The catheter (PE-60 tubing), with previously heated tip to form a collar of ~2 mm from the end, was inserted into the bladder through the dome. The tubing was secured to the bladder with a purse string suture (4-0 Ethilon), exteriorized, and the abdominal muscles and skin were closed with wound clips. All animals were returned to their home cages and closely monitored for the brief recovery period for signs of discomfort or stress. No animal in any group (including spinally intact shams) showed signs of stress or discomfort (e.g., irregular respiratory patterns, vocalization to handling, porphyrin staining around the eyes), and thus no analgesics were administered. Two hours after recovery from implantation, the animal was placed in the harness vest used for training to restrict movement, and the exteriorized catheter was connected to an infusion pump and pressure transducer for saline infusion at a rate of 0.25 ml/min using standard protocols (63, 92, 96). Once the voiding cycles were consistent (at least 5 consecutive voiding events with consistent time intervals in between), five consecutive cycles were recorded using a Neuralynx High Density Electrophysiology System (Lynx-8 amplifier, Neuralynx, Bozeman, MT). Various parameters, including baseline pressure, maximum amplitude of contraction, and contraction time, were retrieved for each of the five contractions and averaged for each animal as described previously (63, 90). Note that it is possible for bladder filling to indirectly induce some pressure on the abdomen and thus potentially some discomfort around the incision site, particularly in the noninjured sham group.

Tissue Removal

Following the cystometry recordings, rats were overdosed with the ketamine/xylazine mixture and immediately perfused with heparinized saline followed by a solution of 30% RNAlater (Ambion, Grand Island, NY) in a 1 mg/ml phosphate buffer solution for tissue retrieval (spinal cord lesion site, kidney and bladder tissue). The bladder was removed, blotted dry, and weighed. The left kidney was also removed and along with the bladder placed in a 100% solution of RNAlater for 24 h at −20°C and then flash frozen in liquid nitrogen and stored at −80°C. As described below, bladder tissue was used for measuring connective tissue proteins that provide tensile strength and elasticity (elastin and collagen) using ELISA. Western blots were done to assess the expression of two proteins in the kidney whose presence are indicative of tissue stress or damage (TGF-β and CD11b). Spinal cord tissue containing the lesion area (T8–T10) was also removed and immersed in 4% paraformaldehyde for at least 48 h, followed by a 30% sucrose/phosphate buffer solution with 1% sodium azide for at least 24 h and until the tissue was cut transversely.

After procedures for the fourth and final set of 12 rats were completed, all collected tissues (coded to maintain blindness of experimenter to group identity) were processed together (see procedures for ELISA and Western blots below). After all experiments were complete, the data were decoded and separated into groups (sham, SCI, SCI+QT, SCI+FT) and analyzed using SigmaStat. One-way ANOVA or one-way repeated measures ANOVA with a significance level of $P < 0.05$ was used followed by the Holm-Sidak method for pairwise multiple comparisons according to previously published protocols (90, 91).

ELISA for Elastin and Collagen

The amount of collagen and elastin present in bladder tissue was quantified using a collagen assay and elastin assay (Biocolor, Northern Ireland, UK). For collagen analysis, frozen bladder tissue (kept at −80°C) was thawed and placed in an acid-pepsin solution overnight at 4°C to make the collagen soluble. A 100-μl sample containing the tissue or three reference standards was added to a 1.5-ml microcentrifuge tube, and 1 ml of Sircol Dye reagent was added to each tube. Following 30 min on a shaker, tubes were centrifuged for 10 min and then drained leaving the collagen at the bottom of each tube. An acid-salt wash was then applied to remove any remaining dye. A 250-μl solution of alkali reagent was then added to each tube and vortexed for 5 min. Two hundred microliters of standard, blanks, and

samples were then pipetted into individual wells of a 96-well plate, which was then read at 555 nm using a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA) to determine the tissue collagen content.

For elastin analysis, bladder tissue was placed in 0.25 M oxalic acid and heated to 100°C for 1 h. This was done twice for each sample. A 100-μl volume of sample, standard, or blank was added to a 1.5 ml microcentrifuge tube followed by 100 μl of elastin precipitating reagent. After 15 min, tubes were centrifuged and drained leaving the α-elastin at the bottom of each tube. A 1-ml volume of dye was then added to each tube and placed on a shaker for 90 min. Tubes were then centrifuged and drained again. Then, 250 μl of dye dissociation reagent was added to each tube and vortexed. Each tube was then revortexed 10 min later. An amount of 200 μl from each tube was then placed in a 96-well plate, which was read at 513 nm using the Spectramax Plus microplate reader to determine the tissue elastin content.

Western Blots for TGF-β and CD11b

Expressions of the proteins TGF-β and CD11b were analyzed with Western blotting following our previously published protocols (40). The left kidney was sectioned (to include both cortex and medulla in the sample) on an ice tray and homogenized in ice-cold lysis buffer [50 mM Tris·HCl (pH 8.0), 200 mM NaCl, 50 mM NaF, 0.3% Triton X-100, 1 M DTT, 1 M benzamidine, 100 mM Na-orthovanadate, 100 mM PMSF] and protease inhibitor (78425, Halt protease inhibitor single-use cocktail, Thermo Scientific). For the protein assay, the Bio-Rad protein assay reagent was used. Protein estimation was done in a photometer at 590-nm absorbance. Samples were loaded with 4× loading buffer (dye) by adjusting the proportion according to the value derived from the estimation. Gels were run in mini-protean gel tanks (Bio-Rad mini-protean tetra system) in 1.5-mm precast gels (456-1083, mini-protean TGX gels, Bio-Rad) at 100 V for 1.5 h. The running buffer was 1× Tris-glycine-SDS buffer. A protein ladder (161-0374, Precision plus protein standards, dual color, Bio-Rad) was used for the band-level detection. Protein was transferred overnight at 30 A on nitrocellulose membranes (162-0115, Bio-Rad) in the transfer buffer (1× Tris-glycine-methanol-SDS buffer). Membranes were then blocked in 3% nonfat dry milk, a TBST solution was applied overnight, and then the membranes were washed the next day with TBST. The membranes were then cut at the level of 75 kDa, the upper portion was incubated in rabbit anti-CD11b (75476, Abcam), and the lower in rabbit anti-TGF-β (3711, Cell Signaling). The membranes were incubated overnight in primary antibody at 1:1,000 dilution (diluted in TBST and 3% nonfat dry milk solution) on a mechanical shaker in a cold room. The following day, membranes were incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (7074, Cell Signaling) for 1 h at room temperature after washing in TBST buffer three times for 5 min each on a mechanical shaker. The membranes were treated with HRP antibody detection reagent (HyGlo chemiluminescent) and exposed to autoradiography film (HyBlot autoradiography film, Denville Scientific). β-Actin (4967S, Cell Signaling) was used as a loading control. After exposure for principal proteins, the membranes were stripped and reprobed with rabbit anti-β-actin antibody at 1:1,000 dilution.

All the samples were run at least three times, and the bands from all the tests were analyzed by inverse densitometry using ImageJ 1.47 (version 1.47, National Institutes of Health) according to our previous protocols (40). Each individual value was normalized by subtracting the background and dividing the value with corresponding β-actin values (minus background). The values obtained from the ImageJ program were then analyzed for the various group comparisons. For the calculation, final raw values for the proteins were obtained after subtracting the background and dividing the result with the loading control. Each value obtained from the calculation for SCI and training groups was normalized with the mean value of shams (i.e., sham controls were set to a value of 1). Statistical analysis included one-way ANOVA with Tukey's honest significant difference post hoc *t*-tests. All values are expressed as means ± SE. A P value of ≤0.05 was considered statistically significant.

Histology

The lesion site tissue was sectioned on a cryostat (Leica CM 1850) at 30-μm-thickness and stained with Luxol fast blue and cresyl violet (Kluver-Barrera method) per established protocols (41, 90). Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI) and a Nikon E400 microscope were used to obtain measurements for quantification of the lesion epicenter (based on total lesion area) as previously described (41, 91). The percent white matter sparing was determined by dividing the white matter remaining at the epicenter by the average area of white matter present in more intact sections. The intact area of white matter for a given region is estimated by averaging measures from two sections 2 mm rostral and two 2 mm caudal to the epicenter. To compare white matter sparing with bladder function, a multiple regression analysis was performed.

RESULTS

A total of 46 male rats were randomly divided into 3 groups (SCI, SCI+QT, SCI+FT) at 2 wk postinjury (2 of the initial 48 died due to complications following the injury). Post hoc analysis of the group data for contusion parameters (kilodyne force and impactor displacement), 4-day urine volume, and 2-wk BBB locomotor score reveals no significant differences with respect to the injury itself and functional outcomes between the groups before the initiation of training (Fig. 2).

Data from the weekly metabolic cage CLAMS system was generated by Oxymax computer software and saved for post hoc analysis. To avoid potential week-by-week variability, 24-h data obtained from two separate time points were averaged for analysis, including two baseline measures preinjury, each of the 2-wk measures postinjury/pretraining, two midway training time points (*weeks 3* and *4*), and the last two training week time points [*weeks 8* and *9*-*week 10* (12 post-SCI) was terminal assessment week]. The data for total 24-h urine volume, average volume per void, total number of voids in 24 h, and total 24-h water intake are presented in Fig. 3. A representative example of the 24-h micturition cycle for an SCI and an SCI+QT rat at four different time points (before injury, after injury, midtraining time point, late in training time point) is provided in Fig. 4. Note that the SCI-induced increase in production of urine after injury was not due to an increase in water intake (compare with preinjury control values for all groups in Fig. 3), as we have shown previously (91). The CLAMS data, when considered in their entirety, suggest that the higher volume per void for the SCI control group after the 9-wk training period could be a compensation for the higher 24-h production of urine (polyuria) for that group. The terminal awake cystometry data (Fig. 5) are consistent with these findings, as the maximum amplitude of contraction was significantly higher for only the SCI nontrained group relative to shams, i.e., a higher void volume requiring a larger bladder contraction to empty (#, Fig. 5). Note, however, that the trained SCI animals as a group were also significantly different from the nontrained SCI animals (**$P < 0.01$), but, individually, only the SCI+FT group was significantly different (*$P < 0.01$). No other differences were found between groups relative to shams (intercontraction interval and peak pressure; not

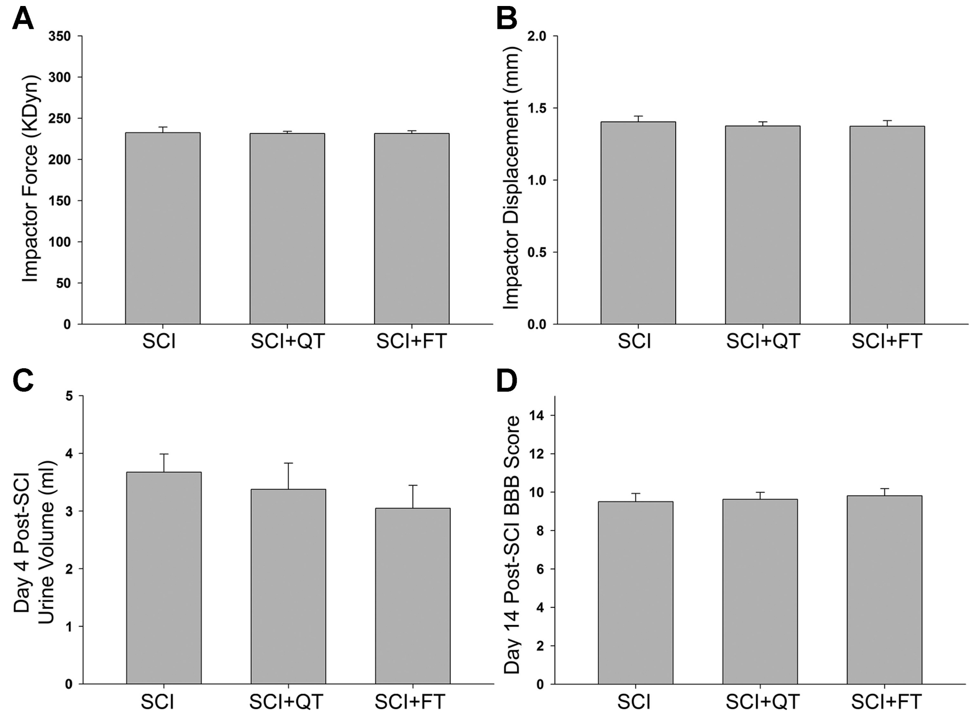

Fig. 2. Pretraining group data. Post hoc analysis of the computer-generated IH impactor parameters [force (*A*); displacement (*B*)] indicate that there were no significant differences among the 3 groups of animals for extent of injury (as anticipated with randomization). The data in *A* and *B* are consistent with the lack of functional differences in the bladder (*C*; maximum residual volume collected from each rat on *day 4*) and overground locomotion (*D*) among the groups before the initiation of training, as it is known that larger residual volumes and lower Basso-Beattie-Bresnahan open-field locomotor test results are reflective of greater injury severities (91). SCI (nontrained); SCI+QT (quadrupedal trained); SCI+FT (forelimb-only trained). Values are means ± SE.

shown). No differences were found in bladder weight and the ratio of elastin-to-collagen between SCI groups, although bladder weight was significantly higher for all groups at 12 wk post-SCI ($P < 0.05$; Fig. 6).

Expression of the proteins TGF-β and CD11b were analyzed with Western blots using the left kidneys from shams and the three SCI groups. For TGF-β, two different bands were detected: one protein band located at 25 kDa and the other at ~50

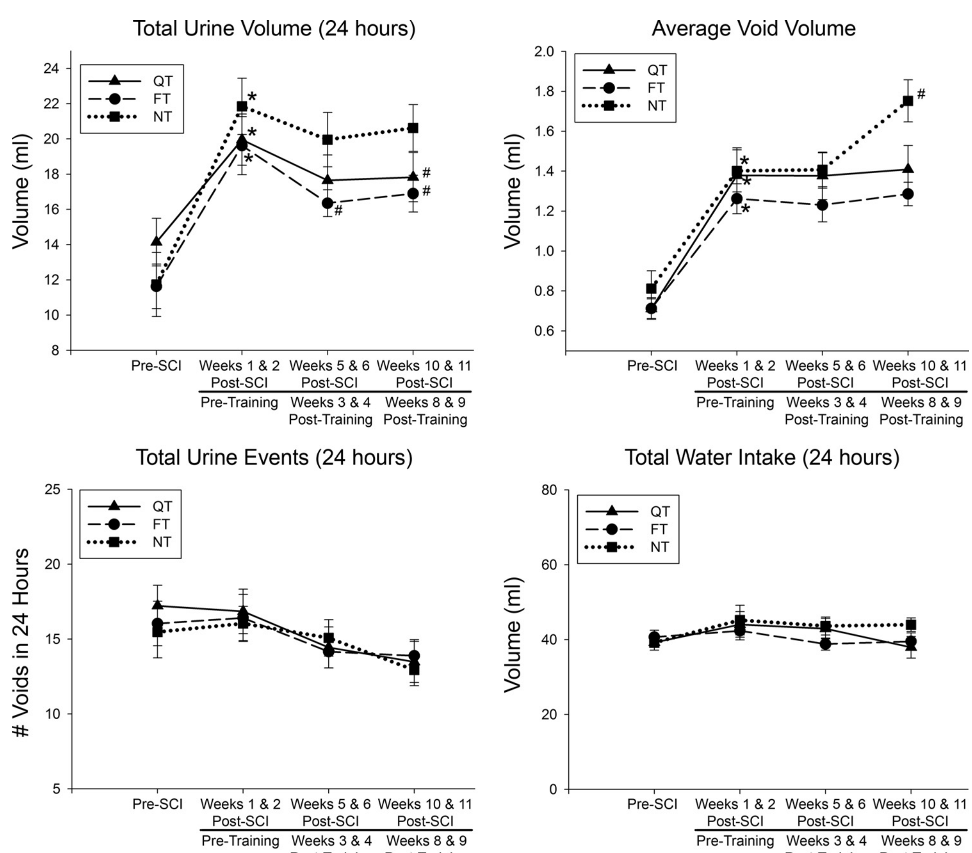

Fig. 3. SCI-induced polyuria. Total urine volume increased significantly post-SCI (**P* < 0.05) but was significantly lower after 9 wk of either SCI+QT or SCI+FT but still significantly above baseline (#*P* < 0.05). The average volume per void was also significantly greater after SCI (**P* < 0.05), although posttraining only the nontrained (NT) SCI group had a further increase in average void volume (#*P* < 0.05). There were no differences found in the total number of urine events or water intake, suggesting that the higher voiding volume for the NT group after training compensated for the higher 24-h production of urine. Note that the surgical sham group was not subjected to this time-consuming testing as each animal served as its own control (i.e., pre-SCI baseline). Values are means ± SE.

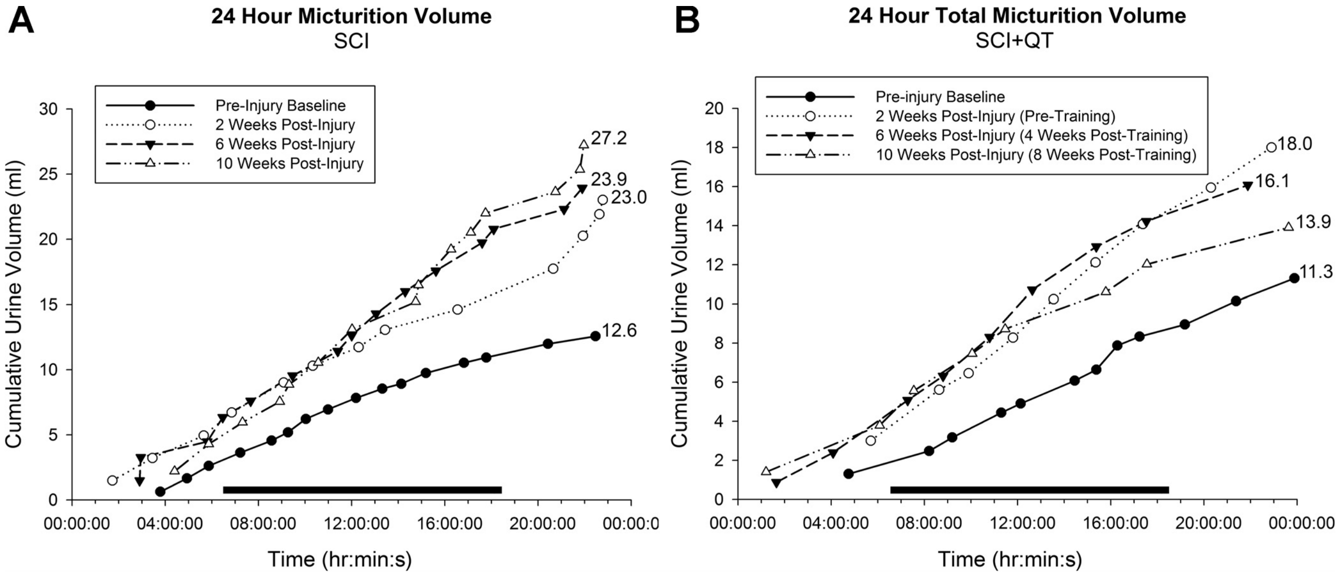

Fig. 4. Twenty-four-hour micturition cycle. Shown are representative total 24-h urine events measured with the CLAMS system for 2 rats, 1 from the SCI control group (*A*) and 1 from the SCI+QT group (*B*). Each plotted line represents a different time point of the experiment; preinjury baseline (●); 2 wk postinjury (pretraining; ○); 6 wk postinjury (4-wk posttraining time point in *B*; ▲); 10 wk postinjury (8-wk post-training time point in *B*; △). Each individual symbol represents a single urine event that is a cumulative total over the 24-h period. The horizontal bar represents the 12-h phase when the housing facility lights are off (active period). Note that the majority of urine events occur during the active phase.

kDa. These bands represent mature TGF-β and TGF-β precursors (known to produce a band between 45 and 65 kDa), respectively, per the manufacturer's datasheet. The 25-kDa bands (mature) of TGF-β were analyzed, and a comparison between the expressions in sham vs. SCI (nontrained) animals revealed a significant decrease in kidney TGF-β levels following chronic SCI (Fig. 7*B*). For the SCI+QT and SCI+FT trained groups, the protein levels were not significantly different from shams ($P > 0.05$). In contrast, the kidney CD11b levels (location at ~160 kDa per the manufacturer's datasheet) increased in density at the 12-wk postinjury time point (SCI) and remained significantly higher, relative to shams, in the SCI+QT/SCI+FT training groups (Fig. 7*C*).

Histological analysis of white matter sparing at the lesion epicenter as well as estimates of the total lesion volume revealed no significant differences between the three groups of SCI rats. A summary of the mean data is presented in Fig. 8. Total lesion volume ranges for the three groups were 9.1–15.6 mm^3 (SCI), 9.6–14.7 mm^3 (SCI+QT), and 9.0–14.9 mm^3 (SCI+FT). Epicenter percent white matter sparing ranges for the three groups were 4.5–32.4% (SCI), 6.1–42.2% (SCI+QT), and 5.9–44.1% (SCI+FT). To further explore the possi-

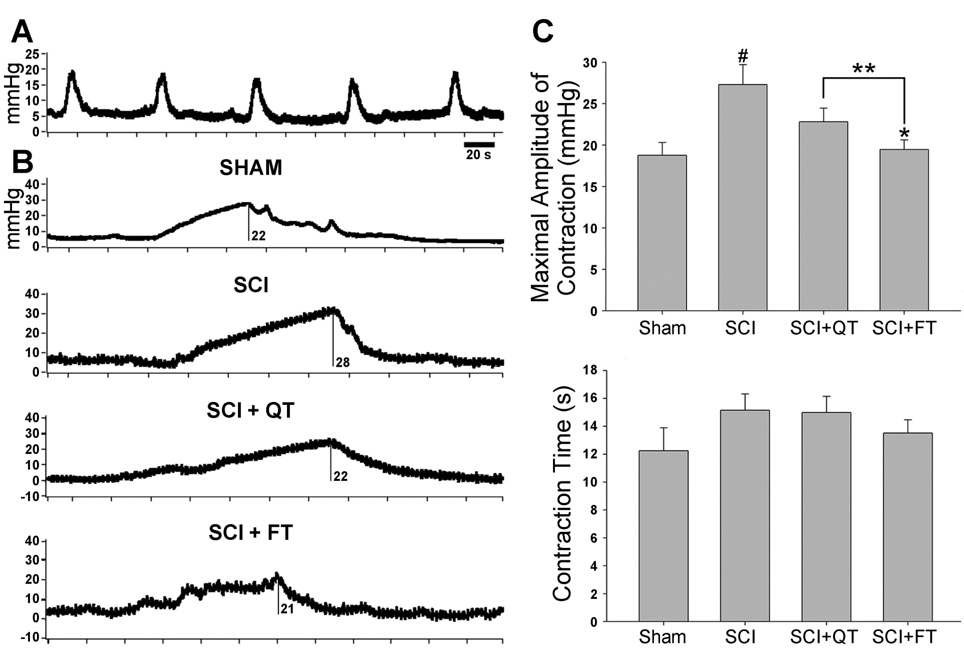

Fig. 5. Terminal awake cystometry. Shown are raw recordings of fill/void cycles from each group of animals. In *A*, 5 full fill/void cycles are shown for a QT animal. In *B*, a representative example of 1 fill/void cycle (note the scale bar) is provided for each group (different QT animal from *A*), and the maximum amplitude values are shown (mmHg). The group means of the averaged data is graphed in *C*. Significant group differences are shown relative to shams (#) and relative to nontrained SCI animals (*, **). Values are means ± SE.

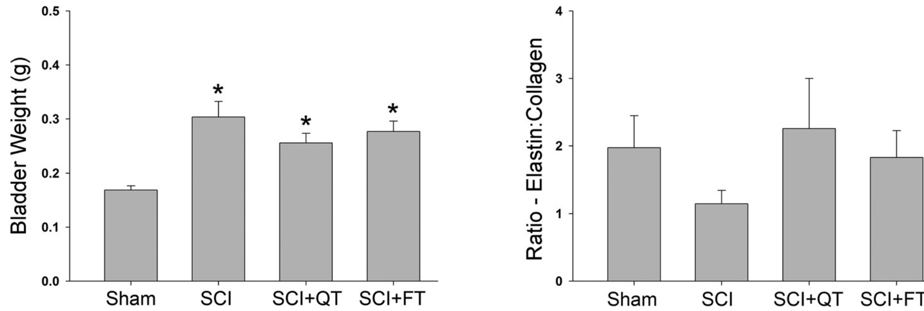

Fig. 6. Bladder tissue analysis. Bladder weight but not the ratio of collagen to elastin differed significantly at the 12-wk post-SCI time point relative to noninjured surgical shams. No differences were found between the trained and nontrained SCI groups. Values are means ± SE.

bility of relationships between injury severity and bladder function, a multiple regression analysis was done, and no relationship was found ($P > 0.05$) between epicenter white matter sparing and bladder outcomes (including voided volume) within each training group, and the training effect is independent of lesion variability.

DISCUSSION

The results of the present study demonstrate an impact of exercise training on multiple post-SCI-induced impairments related to various aspects of urinary tract function using metabolic cage frequency-volume measurements of voiding and awake cystometry. Improvements in both the SCI+QT as well as the SCI+FT groups implicate underlying mechanisms beyond repetitive sensory information from the hindlimbs driving spinal network excitability of the lumbosacral urogenital neural circuitry. Furthermore, preliminary evidence for the potential impact of exercise training on the upper urinary tract in addition to the lower urinary tract underscores overall health benefits of activity-based training on the entire urinary system within the SCI population.

SCI-Induced Polyuria

Metabolic cage data indicate that following SCI, mean 24-h urine volume and the average volume per void increased in all groups of animals, a finding consistent with our previous study demonstrating SCI-induced polyuria for a wide range of spinal lesion severities (mild to severe) (91). After 10 wk of activity-based training, total urine volume and average volume per void were significantly lower in both the SCI+QT and SCI+FT groups relative to the SCI group, although the trained group values were still significantly above preinjury baseline. The further increase in average void volume at the later time points could be reflecting better efficiency (i.e., lower residual volumes), which would be consistent with the significantly higher maximum contraction amplitudes for the SCI rats relative to the sham animals as revealed with terminal cystometry. Note that the metabolic cage procedure that was done weekly does not give a measure of residual volume. Future studies would need to involve a time course for awake cystometry with a chronically implanted bladder catheter to further address these novel findings. The benefits regarding the duration of intense physical activity with 60 consecutive min of training (current and previous data on locomotor and nonlocomotor systems including the bladder) (90) but not 30 min of stepping as we have shown previously for SCI-induced polyuria (91), are consistent with a study on locomotion using a rat spinal transection model showing dependence of functional recovery on the number of repetitions of the weight-bearing stepping activity (15).

Potential mechanisms for improving SCI-induced bladder dysfunction with activity-based training include general exercise effects that impact target organ-neural interactions or potential plasticity of the spinal bladder reflex circuitry (such

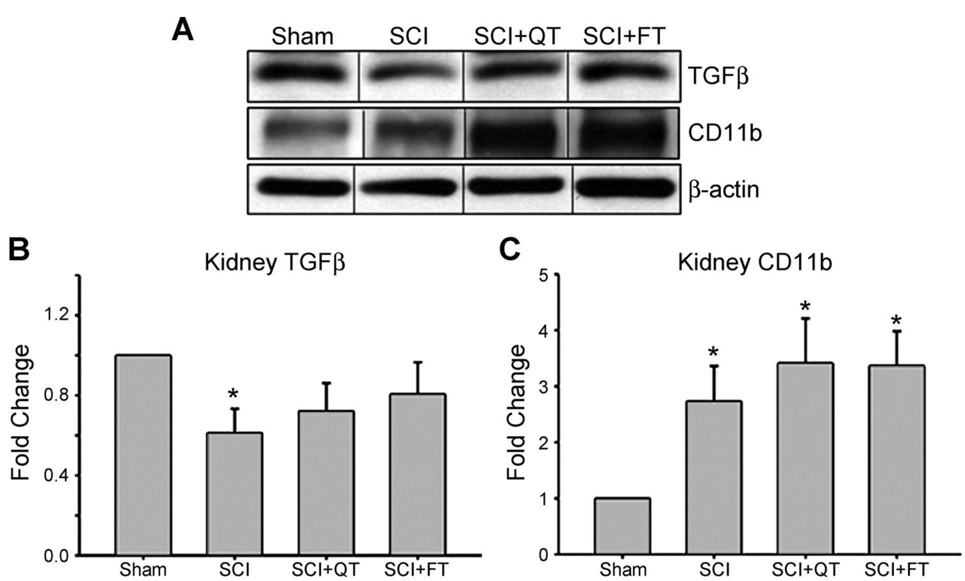

Fig. 7. Kidney transforming growth factor (TGF)-β and CD11b levels. *A*: representative examples of kidney TGF-β and CD11b expression levels. *B*: both SCI+QT and SCI+FT groups had similar expression of TGF-β relative to surgical sham controls. Note that although the SCI trained group levels showed a trend toward sham, they were not significantly different from the nontrained SCI group. *C*: expression of CD11b was significantly higher relative to shams for all SCI groups, regardless of training. Values are means ± SE. *Significant difference ($P <$ 0.05).

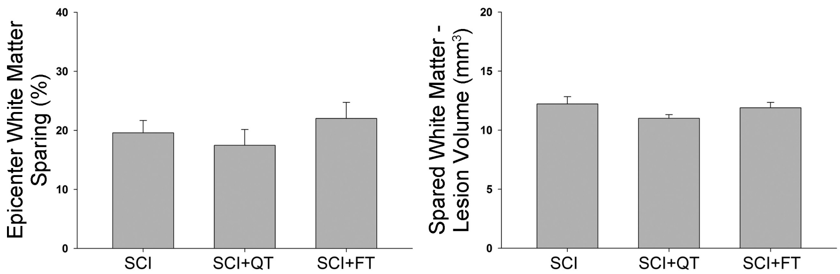

Fig. 8. Lesion histology. No differences were found between the trained and nontrained groups of injured animals at the 12-wk post-SCI time point. Values are means ± SE.

as changes in the properties of afferent neurons in the bladder receptors and/or cell bodies and/or terminals within superficial laminae of the dorsal horn). For example, one likely contributor to the observed SCI-induced polyuria and subsequent alterations in bladder function is hyponatremia (a decrease in the level of serum sodium), which can often occur due to high water content in the blood (55). Hyponatremia is often associated with hyposmolality (a decrease in blood osmolality). One of the easiest ways for the body to increase the level of sodium and increase plasma osmolality is to increase water excretion through urination. Several studies have shown that hyponatremia develops following SCI (10, 33, 70). The trigger that the body uses to increase water excretion in response to hyponatremia and/or hyposmolality is to decrease the release of antidiuretic hormone (ADH; also known as vasopressin) from the posterior pituitary gland (neurohypophysis). When plasma ADH levels are low, the body excretes more water. Importantly, a number of studies involving able-bodied human subjects indicates that exercise can increase plasma ADH levels (45, 87, 88). Given that exercise and LT are rehabilitative therapies that many SCI individuals receive, there is potential for improvement in urological function as long as sufficient amounts are provided [recall that our animal data indicate improvements with 60 but not 30 min (91) per day of intense training]. Our initial data with human SCI research participants (at least 2 yr postinjury) indicate significant improvements in bladder function (as assessed with cystometry) following 80 sessions of LT or LT plus stand training for 60 min/day 5 days/wk (48). Note that if polyuria following SCI is triggered by hyponatremia and/or hyposmolality, then there should be a decrease in the plasma ADH levels post-SCI. Recent data from our laboratory using male rats with a T9 contusion indicate that serum ADH levels decrease significantly 2 wk after severe SCI (unpublished observations), which is consistent with our metabolic cage data showing significantly elevated total 24-h urine volume at 2 wk (Fig. 3). Further studies are in progress. Anecdotally, SCI individuals often report limiting fluid intake to decrease the number of catheterizations over a 24-h period. However, this lack of fluid intake can lead to other metabolic consequences (77). Although polyuria is common after SCI (56), few studies have investigated the mechanisms underlying this condition.

An additional potential mechanism for the observed changes in urological function with LT could involve somato-pudendal reflex interactions such as the flexor and extensor reflex circuitry interactions with external urethral sphincter reflexes as described previously by Tai et al. (81). Motor and autonomic output of the spinal cord is driven in large part by afferent input and local or propriospinal circuitry emphasized after SCI conditions (7, 12, 17, 34, 44), which create the potential for interactions and the triggering of some plasticity within the lumbosacral circuitry to the bladder. For example, multiple sensory inputs from the periphery during locomotion, particularly limb loading (28) and stepping rate (31), provide information to these networks to improve stepping (16, 21, 22, 24, 25). These interactions, however, would not explain the improvement observed with the forelimb exercise group of rats receiving 60 min of daily training. It is conceivable, however, that intense repetitive forelimb exercise with sufficient spinal network excitability via residual supraspinal input induces a net improvement in functional reorganization of the lumbosacral neural circuitry (14, 58, 67, 78). In individuals with an incomplete spinal cord injury, there is some facilitation of the lower extremity muscles in those subjects when walking with reciprocal arm swing vs. without (walking while holding on to parallel bars) (8, 85). Specifically, the presence of some residual intact long propriospinal interenlargement pathways that mediate interlimb coordination for locomotor function in the rat (72) could induce adaptive changes to neural networks within the lumbosacral spinal cord, such as those controlling the bladder. However, given the severity of injury (22.0 ± 2.7% white matter sparing at the epicenter for the forelimb-trained SCI group) and the fact that there was little or no hindlimb activity in the FT group during training and no air-stepping, the probability for the occurrence of such interactions is likely low. Also note that the animals with the most spared white matter did not have the best recovery of bladder function. Thus, although load-bearing on the hindlimb has been shown to be of critical importance for stepping (82), other systemic factors may be vital contributors for improving bladder function with task-specific training based rehabilitation.

Kidney Findings

The results from the present study demonstrate that after a clinically relevant spinal contusion injury, there is a significantly lower level of TGF-β expression in the kidney (NT group) relative to shams. Since TGF-β controls T cell activation and abolition of TGF-β causes gradual infiltration of leucocytes into multiple organs (37, 62, 65), our finding may indicate the presence of an altered immune response in the kidneys during the chronic phase after SCI. In addition to lower TGF-β levels, there was a corresponding rise of CD11b expression in the kidney after chronic SCI (SCI group relative to shams). TGF-β has a known inhibitory effect on CD11b expression (5), which may explain the post-SCI increase in CD11b activity. Note that activation of macrophages, which play a role in both injury and repair in kidney tissue (27), is

required for the synthesis of TGF-β, so a rise in CD11b expression may also indicate activation of endogenous macrophages for tissue homeostasis.

In both SCI training groups, the level of TGF-β expression was not significantly different from the levels in surgical sham animals, indicating the possibility of immune homeostasis and maintenance of renal health (normalized glomerular filtration rate and kidney function) and decreased susceptibility to infection, which would impact the quality of life for the SCI population. Exercise has been shown to reverse negative immune alterations (35), including after SCI (61), but the time since injury and the ideal starting point of training as well as the duration and intensity (57, 89) need further consideration to optimize the most effective strategy not just for urinary tract function but functional recovery in general.

Perspectives and Significance

Bladder dysfunction after SCI is rarely studied in experimental animals yet is overwhelmingly the most significant concern for those suffering from SCI, and urological complications result in significant morbidity and mortality. Importantly, even small improvements in bladder function can have a tremendous impact on these individuals' continual health and quality of life. Results from this animal study provide some initial clues about potential underlying mechanisms regarding our findings on the effects of activity-dependent plasticity induced by LT after chronic SCI on nonlocomotor systems (i.e., bladder function).

Conclusions

Our studies to date indicate that activity-based training can influence urological outcomes, which are of great importance to persons with SCI. The positive benefits of exercise on bladder function post-SCI are likely indirect. These novel findings suggest that physical activity after SCI could translate to significant quality of life gains.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.H.H. and S.J.H. contributed conception and design of research; C.H.H., L.R.M., J.D.F., J.E.A., P.P., and A.N.H. analyzed data; C.H.H., L.R.M., P.P., A.N.H., and S.J.H. interpreted results of experiments; C.H.H., L.R.M., J.D.F., J.E.A., and P.P. prepared figures; C.H.H. and P.P. drafted manuscript; C.H.H., L.R.M., P.P., A.N.H., and S.J.H. edited and revised manuscript; C.H.H., L.R.M., J.D.F., J.E.A., P.P., A.N.H., and S.J.H. approved final version of manuscript; L.R.M., J.D.F., J.E.A., P.P., and A.N.H. performed experiments.

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Research Article

Training-Induced Functional Gains following SCI

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We previously demonstrated that daily, hour-long training sessions significantly improved both locomotor (limb kinematics, gait, and hindlimb flexor-extensor bursting patterns) and nonlocomotor (bladder function and at-level mechanical allodynia) functions following a moderate contusive spinal cord injury. The amount of training needed to achieve this recovery is unknown. Furthermore, whether this recovery is induced primarily by neuronal activity below the lesion or other aspects related to general exercise is unclear. Therefore, the current study objectives were to (1) test the efficacy of 30 minutes of step training for recovery following a clinically relevant contusion injury in male Wistar rats and (2) test the efficacy of training without hindlimb engagement. The results indicate that as little as 30 minutes of step training six days per week enhances overground locomotion in male rats with contusive spinal cord injury but does not alter allodynia or bladder function. Thirty minutes of forelimb-only exercise did not alter locomotion, allodynia, or bladder function, and neither training protocol altered the amount of in-cage activity. Taken together, locomotor improvements were facilitated by hindlimb step training for 30 minutes, but longer durations of training are required to affect nonlocomotor systems.

1. Introduction

From lower vertebrates to humans, it is known that locomotor activity is generated by spinal neuronal circuits referred to as the central pattern generator (CPG) [1]. Studies in lower vertebrates have been useful for modeling how the CPG performs in the absence of supraspinal and afferent feedback as well as determining the roles of neurotransmitters and afferent stimuli (load, speed, and perturbations). Within the context of spinal cord injury (SCI), central pattern generation has become a conceptual basis for locomotor training after injury [2, 3].

In rats, cats, and humans, a large amount of spontaneous recovery can occur following SCI, and this recovery is closely related to white matter sparing [4–6]. Specifically, this recovery has mainly been attributed to spared fibers in the ventral and ventral lateral funiculi where the rubrospinal, reticulospinal, and vestibulospinal tracts are located [7, 8]. Yet, the recovery achieved via training is generally specific to the task practiced, for example, stand, step, or swim training [9, 10]. The amount of activity imposed on the limbs is also a crucial component of locomotor rehabilitation. Importantly, step training on a treadmill using body weight support should provide a high number of repetitions to facilitate motor learning [11]. Recovery potential therefore is a function of (1) amount of sparing/injury severity, (2) task specificity, and (3) amount of activity (activity dependent plasticity). Other aspects of step training or general exercise may also contribute to SCI recovery, including environmental enrichment, intermittent hypoxia, and general improvements in body strength and psychological well-being. More studies are needed to determine the efficacy and mechanisms of training on functional outcomes (including locomotion, allodynia, and autonomic functions) after incomplete contusions in combination with appropriate control groups, kinematics, and overground locomotion (for review see [12]).

In humans, complete cord transection is rare. Therefore, a clinically relevant rat contusion model of SCI may provide

very useful information for the study and translation of locomotor training. The rat model exhibits similarities to human SCI progression [13]. Research with spinally complete and incomplete rodents has affirmed central pattern generation and identified important aspects of training and SCI locomotion [11, 14, 15]. While it is clear that training influences aspects of treadmill locomotion post-SCI, basic research studies have yielded conflicting outcomes regarding overground locomotion [16–19]. Furthermore, very little is known about the efficacy of step training on nonlocomotor functions.

We previously demonstrated that 60 minutes of training 7 days per week significantly improved both locomotor and nonlocomotor functions, such as open field locomotion, hindlimb kinematics, at-level mechanical allodynia, and bladder function in contused rats [20]. In the current study, we (1) tested the efficacy of a 30-minute body weight supported treadmill step training paradigm for the recovery of overground locomotion using qualitative scoring (BBB) and quantitative (kinematic) locomotor tests, (2) determined the contribution of in-cage activity to spontaneous locomotor recovery versus training-induced recovery, and (3) examined bladder function and allodynia (pain response to a nonnoxious stimulus). SCI control groups (nontrain, forelimb) were identically handled, harnessed, and tested. The SCI forelimb trained control group addressed the potential for exercise mediated improvements. Sham (laminectomy only) animals provided a standard comparison throughout the study for each parameter.

2. Materials and Methods

All animal procedures were performed according to the NIH guidelines, and the protocols were reviewed and approved by the Institutional Animal Use and Care Committee at the University of Louisville, School of Medicine. Thirty-eight male Wistar rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) approximately 60 to 70 days old weighing approximately 300 grams were individually housed in an animal room with a 12-hour light and dark cycle. Four animals served as shams (laminectomy controls). SCI animals were randomly divided into three groups before training began as previously described [20]. One group ($n = 14$) was quadrupedally trained; a second group ($n = 10$) served as nontrained controls; a third group was forelimb trained ($n = 10$). Training began at two weeks after SCI for thirty minutes per day, six days per week and continued for six weeks.

2.1. Spinal Cord Injuries. Animals were anesthetized with an intraperitoneal dose of ketamine (80 mg/kg, Ketoset®, Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (10 mg/kg, AnaSed, Lloyd Laboratories, Shenandoah, IA) mixture. Chlorhexiderm scrub cleaned the shaved surgical site. Lubrication was applied to the eyes. The following were administered subcutaneously: 0.5 mL of dual penicillin (PenJect®; The Butler Company; Columbus, OH) single dose perioperatively as a general prophylactic, 5 mg/kg gentamicin (GentaFuse®, Butler Schein, Dublin, OH) once per day for 5 days to prevent bladder infections, 2.5 mg/kg ketoprofen (Ketofen®, Fort Dodge Laboratories, Fort Dodge, IA) twice per day for two days to alleviate postsurgical pain, and 10 mL saline, as previously described [20].

Heating pads maintained body temperature during surgery and throughout the recovery period. The T8 lamina was removed to expose the T9 spinal cord. Spinal clamps were applied to the T7 and T9 spinous processes to stabilize the spinal column. The Infinite Horizon impactor was used to deliver a 210 kdyn impact. Laminectomy shams had the T9 cord exposed but not contused. The muscle was closed with suture, the skin closed with Michel clips, and topical antibiotic applied. Animals were single housed on a 12 : 12 light : dark cycle.

2.2. Behavioral Procedures

Training Paradigm. Training interventions may be less effective when initiated in chronic SCI subjects. However, training interventions initiated too early after SCI may be detrimental to recovery efforts by exacerbating secondary injury cascades [21]. We initiated training at two weeks after SCI after the majority of spontaneous recovery had occurred according to past experience with this injury severity (based on locomotor scores and recovery of spontaneous bladder voiding) [22]. Step training was performed on the Exer3 Treadmill system (Columbus Instruments, Columbus, OH) customized with spring scales for body weight support. The animals were harnessed with lycra vests (Robomedica, Inc., Mission Viejo, CA) and hook-and-loop material and Velcro straps.

Trained animals began quadrupedal step training 2 weeks after SCI surgery, 6 days per week, 30 minutes per day, for 6 weeks, at 22 cm/s. Trainers adjusted the body weight support as needed using manual assistance at the hip flexor region to facilitate proper plantar placement, for example, complete toe extension, no ankle rotation, and incorporation of forelimb-hindlimb coordination with minimal assistance. Rats were encouraged to step independently as they began to gain consistent stepping and more stability without collapsing and dragging their hindlimbs. It is of note that animals were quadrupedally trained, as many studies utilize an upright bipedal training and testing position. The upright position alone can facilitate stepping [23]. Forelimb animals were harnessed and walked with forelimbs on the treadmill while a custom 4 × 6 inch metal platform supported the hindlimbs just above the moving treadmill belt. Nontrained animals were harnessed while a custom platform supported all limbs. For nontrained animals, the 4 × 12 inch metal platform was beside the treadmill belt. One trained, one nontrained, and one forelimb trained animal could be harnessed simultaneously. No body weight support was provided for forelimb and nontrained animals. Sham animals were not exposed to the treadmill system and were handled four times per week (minimum).

Rats were not required to complete the 30-minute session during the first week of training if they exhibited signs of stress, that is, porphyrin staining around the eyes or nose, irregular breathing, or excessive diarrhea. All animals were able to complete the 30 minutes by 6 sessions. Animals were

never stimulated to step by perineum or tail pinching. Noxious stimuli were avoided during training sessions; for example, if an animal had skin abrasion on a paw, animals ceased from training until the issue was resolved, as potentially noxious input may inhibit spinal learning [22, 24, 25].

Locomotor Assessment. An open field locomotor assessment, the Basso-Beattie-Bresnahan (BBB) scale, was used to evaluate hindlimb function in the rats [26]. Once per week, each animal was placed in an open field and tested for 4 minutes by the same two scorers, who were presented with the trained, forelimb, nontrained, and sham animals in random order (blinded to group). The BBB uses a 21-point scale for locomotion, which rates parameters, such as joint movements (0–8), weight support (9–13), and paw placement (14–21). Intact animals demonstrate a locomotor score of 21, whereas animals that exhibit complete paralysis of the hindlimbs are scored as 0. Baseline measurements were collected prior to SCI surgery followed by weekly testing thereafter.

Kinematic Data Acquisition. After the six-week training period the hindquarters were shaved and the bony landmarks on the lateral side of the left and right hindlimbs were marked with permanent marker: iliac crest, greater trochanter, lateral malleolus, and metatarsophalangeal joint (ilium, hip, ankle, and toe). The pad on the plantar surface of the paw was also marked. Each animal was then individually placed in a clear plexiglass runway (in random order). As the animal passed from one end of the tank to the other darkened side, cameras positioned on the side and underneath the tank captured the angles and footfall patterns [27]. 2D overground (unassisted) kinematics were analyzed using the MaxTraq motion analysis system (Innovision Systems, Columbiaville, MI). The iliac crest, hip, ankle (lateral malleolus), toe, and paw were digitized by a blinded observer, and the hip-ankle-toe (HAT) and iliac crest-hip-ankle (IHA) angles were marked to quantify the movement of the hindlimbs during overground stepping. Four animals ($n = 3$ forelimb; $n = 1$ nontrain) could not generate weight bearing steps (BBB = 8; 8 weeks after SCI); these animals did not participate in the RI or PSI tests.

Horizontal Ladder Walk. Animals were additionally tested for fine locomotor control by crossing a horizontal ladder of metal bars (Columbus Instruments, Columbus, OH) [28, 29]. Animals were tested for their ability to correctly place their hind paws while crossing the bars. Forelimb errors were not counted. Animals with more severe deficits (BBB score < 11) were not tested because their limbs drag across the rungs and have a falsely reduced error count. The animals readily crossed the runway with minimal encouragement. Two blinded raters manually counted the number of footfall errors (hind paw/limb slip or fall through the bars). After each crossing, the raters discussed and agreed on the error count. If, at the end of the crossing, the raters did not agree on error count within two errors, the trial was repeated. Footfall error is reported as the mean of three high quality passes (crossed with little or no hesitation and without changing direction; hindlimbs did not drag; raters agreed on error count).

At-Level Allodynia. Behavioral testing of SCI rats for sensitivity to normally innocuous stimuli (touch and gentle squeeze/pressure) was performed using our published grading scale for the scoring of pain-like behavior to trunk stimulation in the rat [30]. Detailed methods also are described in [20]. Once per week, testing sessions commenced at approximately the same time of day (9 am before the start of training). The dorsolateral trunk (T7–T9 dermatomal level) just above the T9 spinal injury level was tested bilaterally for at-level mechanical hypersensitivity to touch and gentle touch/squeeze. Two baseline measurements (at least 3 days apart) were performed before injury for all rats. Throughout the study, all allodynia testing was consistently performed by the same two experimenters, blinded to treatment groups.

At the start of testing, the top of the cage was removed and the animal was allowed to acclimate to the environment for 2 minutes. While in its cage, each animal was stroked at the dorsolateral trunk five times bilaterally with a number 5 paintbrush (1.5 × 0.5 cm bristles; average pressure, 15 g) in an alternating rostral/caudal plane [30]. An interstimulus interval of 1 min between sides was maintained. After each stroking stimulus, the presence/absence of any evoked responses that were indicative of pain was noted: (1) a freezing response (stopping of normal activity and staying still in response to the stimulus), (2) escape (any movement of the animal away from the stimulus probe), and (3) grabbing at or pushing away the stimulus probe with their forepaws. An animal must show an evoked pain-like behavioral response at least 60% of the time in a given session to be considered responsive to the testing stimulus (i.e., an animal responded to at least three of five stimuli/strokes per side) [31]. Responses to brush, if present, were further assessed for threshold values using a set of Semmes-Weinstein monofilaments (20-filament set, 15 of which are in the range of 0.008 g to 15 g; Stoelting Co., Wood Dale, IL). Animals designated as responders to brush (15 g stimulus) were then given a numeric score based on their associated responses to filament testing, receiving a minimum of 4 (4 = freeze, 5 = escape, and 6 = grab/push—as the aggressiveness of the behavioral response increases, so does the score) to a maximum of 10 (see [30] for scoring scale).

Depending on the behavioral response of the animal to brush, an initial filament stimulus was applied by pressing the tip of the filament into the dorsolateral trunk (T7–T9) until it bent. If the animal responded, a lower gram filament was applied to test the animal's sensitivity. In between filament probing, the animal was left alone for a 1 min interstimulus interval. The process was repeated until the lowest gram filament that the animal responded to 60% of the time was determined. If the animal was not responsive to the initial probing stimulus, the next greatest gram filament (and repeated if needed) was used to determine the threshold of the animal's sensitivity.

For those animals not responsive to brush stroke (i.e., evoked pain-like behavioral response to less than 60% of the stimuli—less than three of five strokes), a gentle squeeze/pressure test was conducted to determine if the animal had increased sensitivity to a stronger mechanical stimulus over a wider surface area (which also normally does not provoke

avoidance behaviors and is thus considered innocuous). In this instance, the animal's skin is gently squeezed with a pair of modified Adson tissue forceps (2.0 mm wide tips), which is equivalent to the 60 g Semmes-Weinstein monofilament. Gentle squeeze/pressure was applied to the dorsolateral trunk (T7–T9) five times bilaterally, with an interstimulus squeeze interval of 1 min. As with touch-evoked agitation, any evoked pain-like responses were observed and documented (0 = no response, 1 = freeze, 2 = escape, and 3 = grab/push). After the testing session, animals were scored for their degree of at-level allodynia based on a 10-point scale, with 10 being the maximum score an animal could receive [30]. Scores from each weekly testing were documented for each animal and averaged together to obtain a mean weekly allodynia score for each group.

Transvesical Catheter. After the last training session and all locomotor testing, a transvesical bladder catheter was implanted under 2% isoflurane (similar to [32]). Body temperature was maintained with a circulating water-heating pad. Briefly, the bladder was exposed via a midline abdominal incision through the skin and musculature. A purse-string suture (4-0 Ethilon) was placed in the urothelium of the bladder dome. PE-60 tubing (the tip previously heated to form a collar ~2 mm from the end) was inserted through the bladder dome within the suture limits and secured. The bladder was emptied, and the tubing was passed through the subcutaneous tissue and exteriorized behind the neck [33]. After a 1.5-hour recovery period, the animal was placed in a darkened box for cystometric recordings.

Urodynamic Analysis via Nonstop Transvesical Cystometry. The catheter was connected to an infusion pump and pressure transducer. Normal saline was infused into the bladder at a rate of 0.25 mL/min to evoke voiding contractions [34]. Urodynamic data (voiding and nonvoiding events, voided volumes, and animal movements or spasms) and experimenter notes were recorded on video for offline playback and analysis with Datawave software (http://www.dwavetech.com/). Voiding efficiency was calculated as the percent volume voided per volume saline infused. Cystometrogram (CMG) parameters are the mean of 5 consecutive cycles (which were sampled approximately 15 minutes after the start of saline infusion). CMGs were analyzed for resting pressure (mm Hg), maximal amplitude of contraction (mm Hg; peak pressure minus resting pressure), contraction time (sec), and intercontraction interval (sec).

Activity Meter. The in-cage activity of every animal was recorded using an Opto-M3 infrared activity monitor (Columbus Instruments, Columbus, OH). A cradle equipped with infrared beams, spaced one inch apart, was placed around the cage so that the infrared beams shined across the cage near the floor. The data were collected as ambulatory (number of two consecutive beam breaks; i.e., the animal was moving across the cage) and total movements (number of beam breaks) during the active phase (6 pm–6 am).

2.3. Histology of Lesion Epicenter. Each animal was deeply anesthetized with ketamine/xylazine and perfused transcardially with a solution of normal saline and heparin. The bladder was blunt dissected away from the prostate, blotted dry, and weighed. The spinal lesion area was removed and placed in 4% paraformaldehyde for at least 48 hours, followed by 30% sucrose/phosphate buffer solution with 1% sodium azide for at least 24 hours and until the tissue was cut on a cryostat (Leica CM 1850) at 18 μm thickness and stained with both Luxol fast blue and cresyl violet (Kluver-Barrera method). The lesion area was quantified as previously described [30] using Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI) and the Nikon E400 microscope. Briefly, white matter was divided into four regions (dorsal columns, dorsolateral funiculus, ventrolateral funiculus, and the ventral funiculus) and each area was subdivided into left and right sides. The gray matter was divided into dorsal and ventral regions. The central canal, medial edges of the dorsal horn, and the tips of the ventral horn were used as landmarks for the divisions. The percent of white matter sparing (WMS) was determined by dividing the white matter remaining at the epicenter, .5 mm rostrally, and 1.0 mm rostrally by the average area of white matter present in intact sections. The intact area of white matter for a given region was estimated by averaging together measurements from 2 sections 2 mm rostral to the epicenter.

2.4. Statistics. SCI animals were excluded from analysis if the recorded displacement of the impactor tip was less than 1.0 mm during the injury ($n = 3$); these animals typically have a very mild injury. An additional 2 animals were sacrificed during the first two weeks following injury due to autophagia. This resulted in the following groups: train, $n = 13$; nontrain, $n = 7$; forelimb, $n = 9$; sham, $n = 4$. Analyses were performed using SigmaStat and Microsoft Excel. Levene's test for inequality of variance was performed. One-way repeated measures analysis of variance (ANOVA) (fixed effects) was performed for tests of within subject and between subject effects followed by Bonferroni *post hoc* t-tests for the BBB, allodynia, and activity. One-way ANOVA (fixed effects) was used for cystometry parameters, bladder weight, gait, and kinematics followed by Bonferroni *post hoc* t-tests, significance level $p < .05$. For the regularity index (RI), *post hoc* tests approached significance and were followed by the Mann-Whitney U test. Percent animals with consistent weight support were analyzed with the binomial proportion test. Significance level was $p < .05$. All values reported in the paper are mean ± SD.

3. Results

3.1. Training Significantly Improved Overground Locomotion. All groups demonstrated significant spontaneous recovery from 1 to 2 weeks after SCI. At the initiation of training (2 weeks after SCI) all groups functionally displayed plantar paw placement with occasional weight support (BBB scores: trained: 9.61 ± 1.7; forelimb: 9.5 ± 2.2; nontrained 9.3 ± 2.0). After 3 weeks of training (5 weeks after SCI), trained

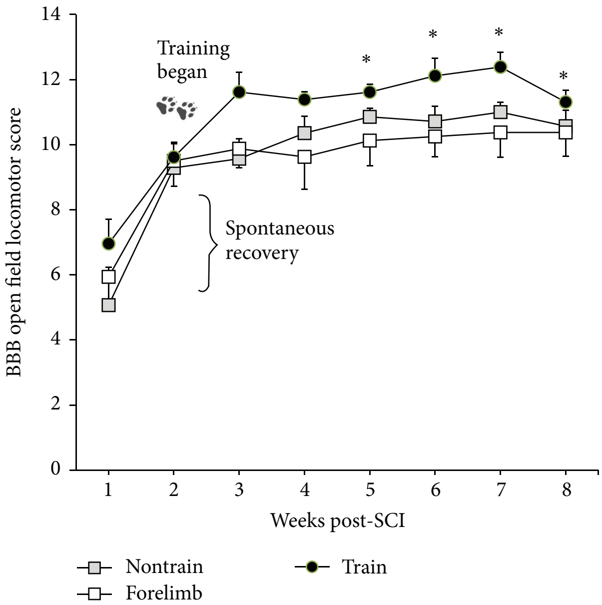

Figure 1: Weekly open field locomotor scoring of trained, nontrained, and forelimb SCI male rats. Statistically significant locomotor recovery occurred from week 1 to week 2 for all groups. At 2 weeks post-SCI training began. Only the trained group had significant increases from pretraining. Shams not shown, BBB = 21 (∗ versus pretraining; W2; repeated ANOVA with Bonferroni *post hoc t*-tests: nontrain $n = 7$; forelimb $n = 9$; train $n = 13$).

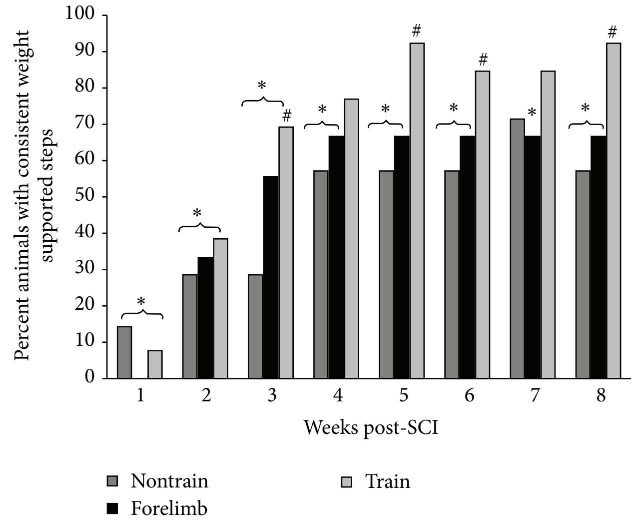

Figure 2: Percent animals with consistent weight support. Significant differences were found between sham and both the nontrained and forelimb groups (∗) at weeks 1–8, except week 7, as well as between the trained and nontrained groups (#) at weeks 3, 5, 6, and 8. No differences were found between sham and trained animals after week 3 (binomial proportion test: nontrain $n = 7$; forelimb $n = 9$; train $n = 13$).

animals' BBB scores were significantly higher compared to pretraining and remained significantly higher through the rest of the study (Figure 1). Forelimb and nontrain controls did not significantly improve from their pretraining BBB score. Consistent weight supported stepping (BBB ≥ 11) is a functional milestone on the BBB scale (which makes each animal eligible to receive a subscore). A significantly higher proportion of animals in the trained group achieved consistent weight support. No differences were found between sham and trained animals after week 3 (Figure 2). However, the trained group was also not significantly different from the forelimb or nontrain groups. Forelimb and nontrain groups had significant proportions of animals *unable* to consistently weight-support as compared to sham throughout the study.

Kinematic measurements of overground locomotion revealed that quadrupedal-trained animals more closely approximated the hindlimb angular movement of shams (no significant differences). However, the trained group was also not significantly different from the forelimb or nontrain groups. Both nontrained and forelimb trained control groups had significantly larger angular excursions of the hip and ankle, as well as significantly larger ankle extension (Figure 3).

The trained animals recovered to BBB scores necessitating the assessment of forelimb-hindlimb coordination. Importantly, although the BBB can be used to assess coordination, we also utilized a more objective assessment of coordination, the regularity index (RI) [35]. The RI is a score of plantar footfall pattern and limb coordination [36], and the trained group scored significantly better than the nontrained group (Figure 4). The plantar stepping index (PSI), which is a ratio of plantar hindlimb to forelimb steps [27], revealed no differences between trained and sham controls, while nontrained and forelimb controls scored significantly lower than shams (Figure 4). However, the trained group PSI was also not significantly different from the forelimb or nontrain groups. Analyses of gait parameters did not detect differences between groups for stride length, stride time, base of support, or toe velocity (data not shown).

The horizontal ladder demonstrated a large difference between injured and sham animals. Sham animals were able to cross the horizontal ladder with only one or two errors (1.1 ± 0.51). Although trained animals displayed improvements in overground locomotion and limb coordination, their ability to control fine placement of the hind paw remained impaired. There were no differences between any SCI group (train 7.67 ± 2.88; forelimb 8.43 ± 2.44; nontrain 7.42 ± 3.80).

3.2. At-Level Allodynia. Quantitative measurements of sensitivity to mechanical stimuli were obtained on two separate occasions prior to injury and then once per week for 8 weeks after SCI in the trained, nontrained, and forelimb groups. Preinjury measurements revealed that none of the groups demonstrated allodynic behavior to innocuous stimuli and were equivalent at baseline. One sham animal vocalized prior to injury and was excluded from this analysis only. The average sham score after surgery was 1.75 ± 2.4. Immediately after SCI, all rats exhibited a moderate degree of evoked at-level allodynia consisting of either freezing, escaping, or grabbing (with or without vocalization) towards a stimulus filament ranging from 15 g to 0.008 g. These pain-related aversion behaviors persisted throughout the course of training for all

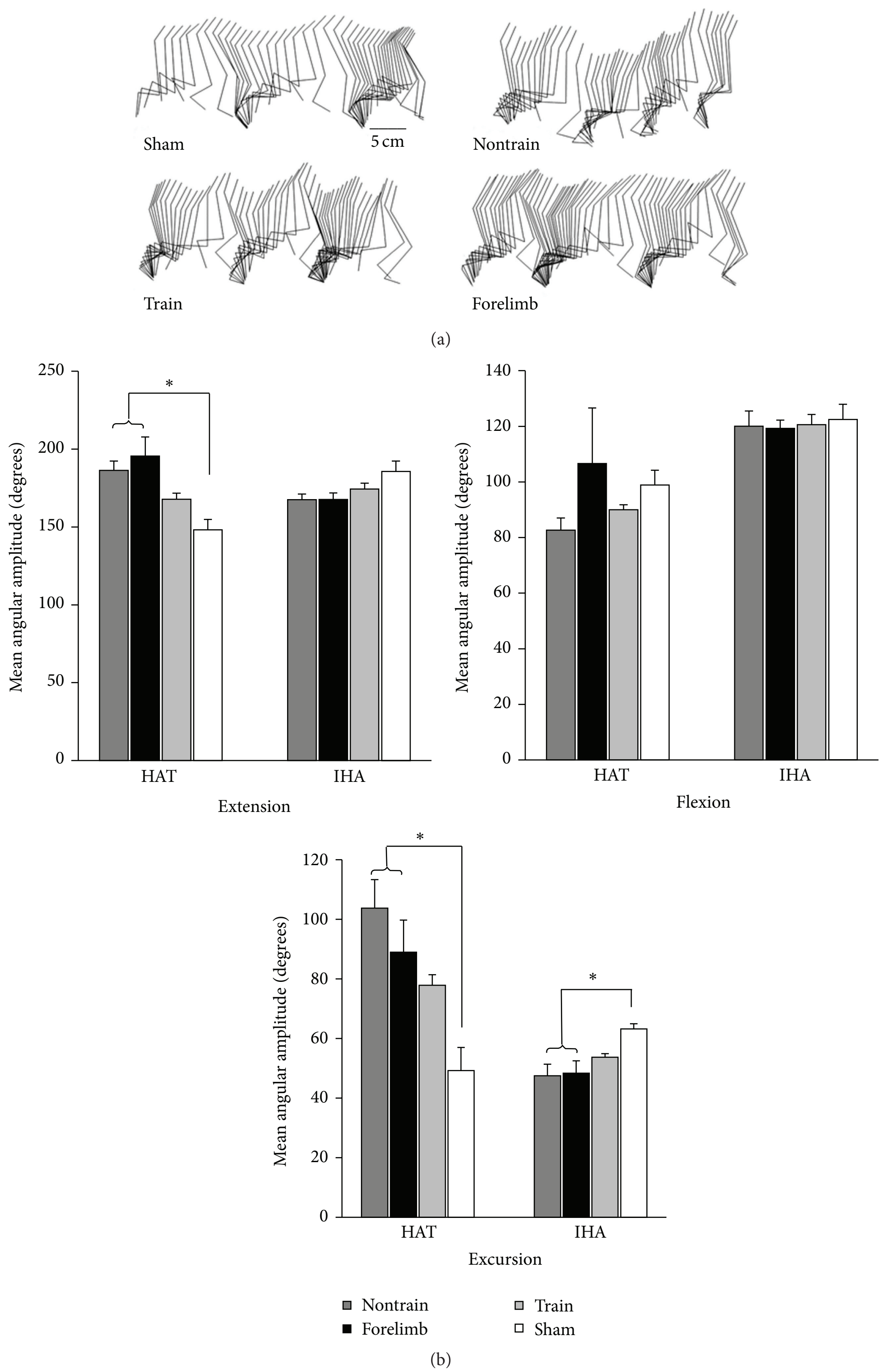

Figure 3: (a) Kinematic illustration of the hindlimb (iliac crest, greater trochanter, knee, lateral malleolus, and metatarsophalangeal joint) during stepping. (b) The maximum (extension) and minimum (flexion) angles as well as excursion (range of motion) of the hip-ankle-toe and iliac crest-hip-ankle angles were calculated and compared between trained (n = 13), nontrained (n = 7), forelimb (n = 9), and sham (n = 4) (ANOVA with Bonferroni *post hoc* t-tests). Significant differences were found for ankle extension and hip and ankle excursion (∗ sham versus nontrained and forelimb). Sham was not different from trained.

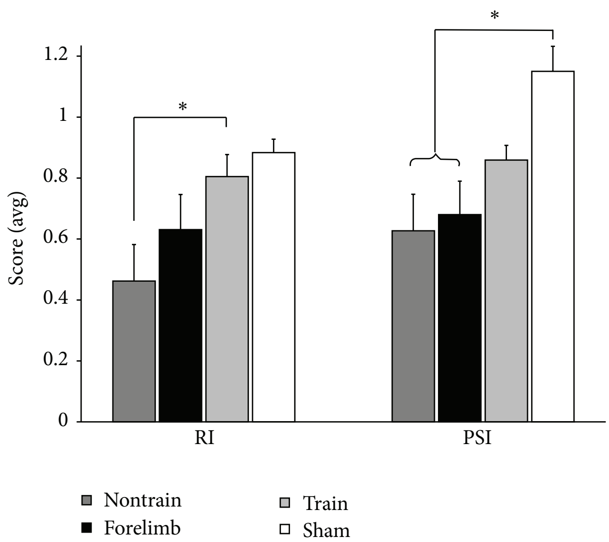

Figure 4: The regularity index revealed a significant difference between the trained and nontrained groups (∗) (Mann-Whitney *U* test). The plantar stepping index revealed a significant difference between the sham and both the nontrained and forelimb groups (∗) (ANOVA with Bonferroni *post hoc t*-tests). Parameters of gait were not significant: stride length, stride time, base of support, and toe velocity. Nontrain $n = 5$; train $n = 13$; forelimb $n = 6$; sham $n = 4$.

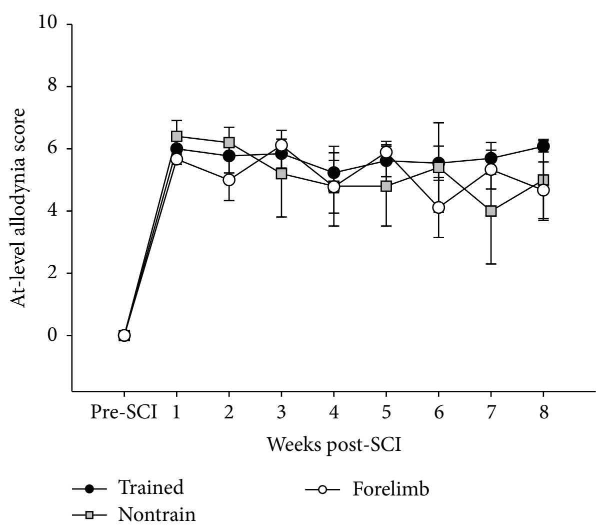

Figure 5: No animals were sensitive to touch or gentle squeeze prior to injury. Immediately after SCI, all rats exhibited a moderate degree of evoked at-level allodynia. There were no differences between groups regarding the course of at-level allodynia.

SCI groups (allodynia score at 8 weeks after SCI: train 5.72 ± 1.44; forelimb 5.19 ± 1.47; nontrain 5.23 ± 2.33) (Figure 5).

3.3. Cystometry and Bladder Weights. Autonomic dysfunction is of high priority for individuals with SCI [37]. We examined a total of 12 urodynamic parameters as well as bladder weights to identify possible treatment effects. Only a few are reported here. Shams were directly compared to SCI groups for bladder weight but not for urodynamics,

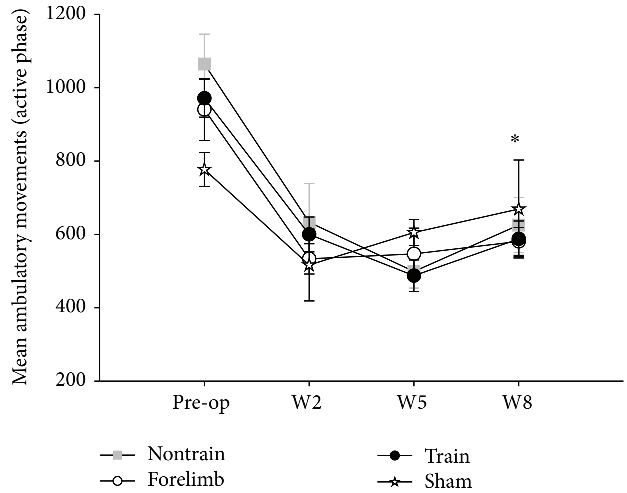

Figure 6: Home cage activity: in-cage activity assessments with an infrared activity monitor show no differences in the amount of ambulatory movements up to week 8 (W8), suggesting that the increased locomotor recovery of the trained group was a result of the step training paradigm and not due to "self-training." All animals significantly decreased activity with time from surgery (∗ preoperation versus W8 for each group; repeated ANOVA with Bonferroni *post hoc t*-tests; nontrain $n = 7$; train $n = 13$; forelimb $n = 9$; sham $n = 4$).

as the fill rate of .25 mL/min causes different physiological outcomes in a very small bladder compared to a bladder twice the normal size. No significant differences were observed between trained, nontrained, and forelimb groups (one-way ANOVA, $p > .05$). Bladder weights of all SCI animals were larger than shams (bladder weight in milligrams: sham 143.3 ± 25.2; train 226.5 ± 52.7; forelimb 287.2 ± 78.9; nontrain 240.0 ± 65.6; one-way ANOVA $p = .013$) [38, 39]. Intercontraction interval (ICI, seconds) was not different between groups (train 130.5 ± 88.8; nontrain 120.0 ± 51.1; forelimb 127.2 ± 77.4). Voiding efficiency was not different between groups (train 89.5 ± 9.8; nontrain 92.3 ± 8.3; forelimb 86.1 ± 15.2).

3.4. Home Cage Activity (6 pm–6 am). Another source of potential locomotor practice is in-cage activity. Home cage activity was monitored during the active phase and revealed that while all groups significantly decreased activity with time, there were no significant differences between any group when measuring ambulatory or total in-cage movements. All groups followed a similar pattern (Figure 6). This finding suggests that home cage activity was not a significant contributor to improved locomotion in the trained SCI group.

3.5. White and Gray Matter Spared. Histological assessment of the injury started with the epicenter and continued 1 mm rostrally. Figure 7 shows representative sections of the contused cord at the epicenter and .5 mm and 1.0 mm rostrally from the injury as well as an intact section. There were no significant differences between any group when analyzing weight gain (each group gained approximately 100 grams), injury parameters, white matter at the epicenter, .5 mm, or

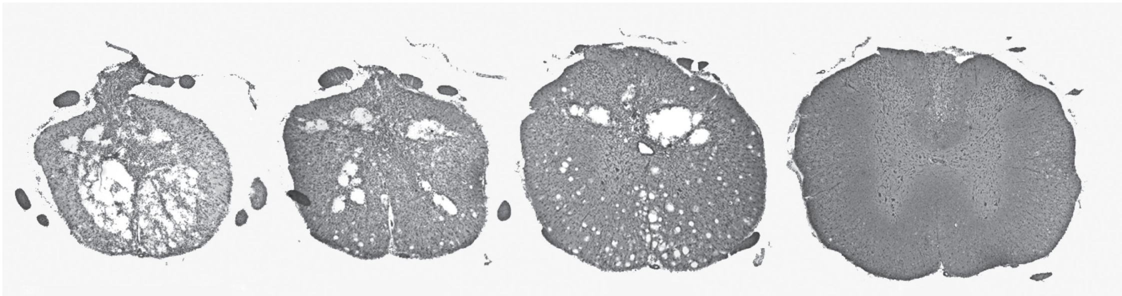

Figure 7: Representative spinal cord segments. Histological assessment did not reveal any differences between groups when analyzing total white or gray matter from the epicenter to 1.0 mm rostrally or when further subdividing the sections into areas of ventral and ventral lateral funiculi (ANOVA: nontrain $n = 7$; train $n = 13$; forelimb $n = 9$).

Table 1

Treatment	Force (kdyn)	Displacement (μm)	WMS% 0.0 mm	WMS% 0.5 mm	WMS% 1.0 mm	Body weight at week 8 (g)
Nontrain	215.57 ± 5.6	1269.4 ± 117.2	9.2 ± 5.1	28.3 ± 8.2	65.0 ± 5.7	417.57 ± 43.8
Forelimb	217.13 ± 4.6	1308.8 ± 140.0	11.4 ± 8.6	27.6 ± 9.0	59.4 ± 11.1	439.7 ± 43.1
Train	217.45 ± 6.6	1269.6 ± 158.9	12.8 ± 7.1	26.9 ± 8.8	59.6 ± 12.7	441.2 ± 35.0

1.0 mm (Table 1). The white matter was further subdivided into ventral and ventral lateral funiculi and gray matter of the ventral horn was also calculated at the epicenter, .5 mm, and 1.0 mm. There were no differences detected between any group when analyzing the ventral portions of the spinal cord. These data suggest that functional differences observed between groups cannot be attributed to differences in the amount of spared white or gray matter. Yet, plasticity within the spared pathways may facilitate functional recovery.

4. Discussion

The method of training used in this study facilitated additional recovery of the trained group (beyond the substantial amount of spontaneous recovery that occurred during the first two weeks following SCI [18, 26, 40]) and is the first to show overground improvements using quantitative kinematic and gait analyses in addition to qualitative open field scoring for contused male rats. The differences in locomotor parameters of trained versus nontrained and forelimb controls could not be attributed to differences in home cage activity, similar to a study comparing spontaneous exercise and enriched environment in which the amount of activity did not correlate to locomotor recovery [41]. Additionally, like other studies using activity based therapies [16, 18, 42], we found that training did not increase white matter sparing, even when analyzing subdivisions of the spinal cord, such as the ventrolateral funiculus. These results suggest that training reinforced or facilitated plasticity within the remaining pathways or lumbosacral circuits rather than promoting regeneration or sprouting of new pathways.

After 6 weeks of training, almost all trained SCI animals had achieved weight supported stepping and degrees of forelimb-hindlimb coordination. In contrast, while many animals in the SCI control groups recovered consistent weight supported stepping, recovery was slower and some animals never regained the ability to weight-support. These data are consistent with stand training in transected cats, which increased the duration of hindlimb standing [43, 44]. In rats with contusion or compression injury, treadmill training improved ankle extension [14] and weight bearing during open field locomotion [16]. Compared to our previous findings [20], as little as 30 minutes of daily locomotor training can improve weight bearing locomotor ability in SCI animals (60 minutes of daily training did not further improve BBB scores).

Many studies perform quantitative kinematic measurements on the treadmill (bipedal and quadrupedal) where the hindlimb is passively extended during stance, the trunk is supported by a harness, and partial body weight support is provided. Here, we used overground kinematic and gait analyses to quantify the training effects on full weight bearing overground locomotion compared to sham and SCI controls. Ankle extension and ankle and hip excursion were normalized by training (more similar to shams). Coordination and plantar paw placement were also normalized (RI and PSI). In cats, treadmill training increases the recruitment of flexor motor pools compared to nontrained cats [6]. Trained SCI cats and rats have also been shown to have greater paw lift and hip flexion, respectively, both allowing a reduction in paw dragging [6, 15, 45]. Our results extend these studies and indicate that training facilitates normal step cycle trajectories during *overground* locomotion by decreasing ankle extension and excursion, increasing hip excursion, and increasing the plantar stepping index.

Step training that provides alternate limb loading and rhythmic repeated steps can promote neural activity and improve EMG patterns and amplitudes [17, 46, 47], which

could explain how ankle extension (during stance), plantar paw placement, and weight support improved with training. Importantly, not all aspects of motor control improved with quadrupedal training. Although training promoted the recovery of overground locomotion, training did not improve the ability to cross a horizontal ladder. The ladder test correlates very well with injury severity [48]. However, this task requires precision paw placement and relies on proprioception [49, 50]. While our training paradigm improved gross locomotion (weight bearing, coordination, etc.), fine proprioceptive movements remained impaired, possibly due to the task specificity of training.

Locomotor training has been reported as having beneficial effects on bladder function in clinical settings [51–53]. Indeed, we previously demonstrated that 60 min of training significantly improved bladder function. However, our results according to cystometry and bladder weights in this study and SCI-induced polyuria in our previous study [54] indicate that 30 minutes of training did not affect bladder function after SCI. Thus, longer training durations should be further investigated for nonlocomotor functional recovery. Indeed, we found that, with 60 minutes of training, both quadrupedal and forelimb training improved the maximum bladder contraction amplitude and reduced SCI-induced polyuria [55].

With respect to sensory function, the emergence of neuropathic pain after SCI significantly impacts patients' quality of life and interferes with functional recovery [56–58]. In the clinical setting, exercise, including treadmill training, can positively influence neuropathic pain [59–61]. We previously demonstrated that 60 min of locomotor training resulted in a significant improvement in at-level allodynia scores compared to the nontrained group. This effect was observed after 3 weeks of training and remained consistent throughout the course of training [20]. Other rodent models of SCI, employing varying degrees of locomotor training intensity and duration, also report attenuations of allodynic responses following either below-level to the plantar aspects of the paws [62, 63] or at-level [64] mechanical threshold testing. In this study, 30 minutes of step training was not sufficient to ameliorate the onset of trunk at-level allodynia after SCI. Differences across studies may be attributed to the spinal location (cervical versus thoracic) and type of injury model (contusion versus compression), gender, magnitude, and intensity of training as well as the region being tested (trunk versus paw). It is not clear whether step training induces a level of resistance to the glabrous plantar aspect of the paw, perhaps influencing mechanical withdrawal thresholds. Overall, the degree of spared spinal pathways, spontaneous recovery, and the rhythmic weight bearing load during stepping, which may promote activation of cutaneous and proprioceptive afferents, may all be driving factors influencing improvements in tactile sensation [63]. A comparison between males and females may also be warranted as the development of pain and the requirements for differential effective exercise protocols may be sex-dependent [65–67].

Enriched environments and spontaneous exercise, such as wheel running, have been shown to improve locomotor recovery after SCI [41, 42, 68]. The possibility exists that exercise could promote post-SCI recovery through more general mechanisms, such as through alterations in inflammatory pathways, a "nursing effect" through neurotrophins, or increased well-being. In this study, we utilized a control group to induce exercise without specific activation of lumbar circuitry. This forelimb trained SCI control group did not show any benefits of exercise. In fact, although not statistically different, the forelimb trained SCI animals scored slightly worse on multiple locomotor parameters, including BBB score, ankle flexion and extension, and ladder errors. These findings suggest that step training improves locomotor recovery through direct activation of lumbar circuitry. In contrast, according to our finding that 60 minutes of forelimb training significantly altered some parameters of urinary tract function [55], other mechanisms must be responsible for these nonlocomotor improvements. In conclusion, a number of factors can enhance or prevent functional recovery after SCI. These factors are related not only to the injury but specific parameters of step training. These factors have been widely varied in experimental SCI and are likely contributing to different conclusions about step training's efficacy in an incomplete rat model of SCI. Our results suggest that 30 minutes of manually assisted step training initiated in the subacute stage of recovery can maximize potential locomotor gains. We found that 30 minutes of quadrupedal step training improved overground weight support, coordination, and ankle/hip range of motion. These improvements could not be directly attributed to lesion variability or home cage activity. We also found that 30 minutes of daily training did not improve precision paw placement, bladder function, or allodynia. While 60 minutes of daily training does not result in even higher BBB scores or an increase in the percentage of animals that can weight-support, longer daily training sessions result in additional benefits to nonlocomotor functions (allodynia and bladder function), which would substantially influence a patient's quality of life.

Competing Interests

The authors declare no conflict of interests regarding the publication of this paper.

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